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Detection of Designer Benzodiazepines in Scottish Sub-populations

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By

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Author's Declaration

"I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution"

Signature _____

Printed name _____

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O'Connor LC, Torrance HJ, McKeown DA. ELISA detection of phenazepam, etizolam, pyrazolam, flubromazepam, diclazepam and delorazepam in blood using Immunalysis® Benzodiazepine Kit. Journal of Analytical Toxicology. 2016 Mar 1;40(2):159-61.

O'Connor LC, Torrance HJ, McKeown DA.2016, *Concentrations of diclazepam and its metabolites found in post-mortem blood.* Poster presented at Scottish Student Forensic Research Symposium. April 2016, Glasgow, Scotland, UK.

O'Connor LC, 2018, Torrance HJ, McKeown DA. *Designer benzodiazepine use in the prison population in Scotland*. 55th TIAFT - SOFT Annual Meeting. January 2018, Boca Raton, Florida, USA.

List of Abbreviations

%CV	Coefficient of Variation percentage
%ME	Matrix Effect
%PE	Process Efficiency
%RE	Percent Recovery
°C	Degree Celsius
µg	Micrograms
µm	Micrometre
6-MAM	6-monoacetylmorphine
ACMD	Advisory Council on the Misuse of Drugs
ACN	Acetonitrile
AM	Ante-mortem
API	Atmospheric Pressure Ionisation
APT	Addiction Prevalence Testing
BNF	British National Formulary
CE	Collision Energy
CEP	Collision Entrance Potential
CI	Chemical ionisation
CNS	Central Nervous System
cps	counts per second
CXP	Collision Exit Potential
D-	Deuterated compound
DFSA	Drug Facilitated Sexual Assault

DHC	Dihydrocodeine
DiH_2O	DiH2O
DMD	Demethyldiazepam or nordazepam
DP	Declustering Potential
DRD	Drug-related death
DTTO	Drug Treatment and Testing Order
EI	Electron Ionisation
ELISA	Enzyme-linked Immunosorbent assay
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EP	Entrance Potential
ESI	Electrospray Ionisation
EWS	Early Warning System
FMS	Forensic Medicine and Science
FTS	Forensic Toxicology Service
g	gram(s)
$GABA_A$	Gamma-Amino Butyric Acid-A
GC-	Gas Chromatography
h	Hour(s)
hCG	Human Chorionic Gonadotropin
HIV	Human Immunodeficiency Virus
HMP	Her Majesty’s Prison
HPLC	High Performance Liquid Chromatography
L	Litre

LC	Liquid Chromatography
LLE	Liquid Liquid Extraction
LLOQ	Lower Limit of Quantitation
LOD	Limit of Detection
LOQ	Limit of Quantitation
m	Metre
M	Molar
m/z	Mass to charge ratio
MDA	3,4-Methylenedioxyamphetamine
MDEA	3,4-methylenedioxy-N-ethyl-amphetamine
MDMA	3,4-Methylenedioxy-methamphetamine
MeOH	Methanol
mg	Milligrams
min	Minute(s)
mL	Milligrams
MMA	Methylmalonic acidemia
MoDA	Misuse of Drugs Act
MP	Mobile phase
MRM	Multiple reaction monitoring
-MS	Mass Spectrometry
-MS/MS	Tandem Mass Spectrometry
msec	Milliseconds
MW	Molecular Weight

ng	Nanogram
ng/mL	Nanogram per millilitre
NH_3	Ammonia
NHS	National Health Service
NHS GGC FD	National Health Service Greater Glasgow and Clyde Forensic Directorate
nm	nanometers
NPDS	National Poison Data System
NPS	Novel psychoactive substances
OST	Opioid Substituted Therapy
OXZ	Oxazepam
PBS	Phosphate-buffered saline
pg	Picogram
pH	potential Hydrogen
PM	Post-mortem
PSA	Psychoactive Substances Act
psi	Pounds per square inch
QC	Quality Control
r	coefficient of correlation
r^2	coefficient of determination
rpm	Revolutions per minute
RSD	Relative standard deviation
RTA	Road Traffic Accident

S/N	Signal to Noise Ratio
SD	Standard deviation
SDC	Scottish Drug Court
SDMD	Scottish Drugs Misuse Database
SFIU	Scottish Fatalities Investigation Unit
SNBTS	Scottish National Blood Transfusion Service
SPE	Solid Phase Extraction
SWGTOX	Scientific Working Group for Forensic Toxicology
t0	Time Zero
*t*BME	tert-methyl butyl ether
THC	Delta-9-tetrahydrocannabinol
TIC	Total Ion Count
TLC	Thin Layer Chromatography
TOF	Time of Flight
UK	United Kingdom
ULOQ	Upper Limit of Quantitation
UNODC	United Nations Office on Drugs and Crime
USA	United States of America
V:V	Volume:Volume
WEDINOS	Welsh Emerging Drug and Identification of Novel Substances
WoRES	West of Scotland Research and Ethics Service

Abstract

Designer benzodiazepine is the term used when referring to benzodiazepines, which have been available for recreational use since the late 2000s. Some designer benzodiazepines are prescribed in other countries and became popular in the UK as a drug of abuse, others were investigated as medicines in the 1960s and 1970s but were never brought to market. A few designer benzodiazepines are novel drugs created solely for the recreational market. Originally sold as “research chemicals” or “legal highs” they circumvented the law by having small structural differences to the traditional benzodiazepines and sold in packages containing the disclaimer “Not for Human Consumption.” The introduction of the Psychoactive Substances Act 2016 (PSA) put new UK legislation in place to control the distribution and manufacture of any compound that is “capable of producing a psychoactive effect,” this captured the designer benzodiazepines as well as other “legal highs”. This legislation works in conjunction with the current Misuse of Drugs Act 1971 (MoDA), thereby legislating against a distinct list of drugs in the MoDA and any drug producing a psychoactive effect in the PSA.

While there is now clarity of the legal status of these drugs, the scale of use in different sub-populations in Scotland, before and after, this legislation is unknown. There is little literature exploring how commonly designer benzodiazepines are detected in post-mortem cases from both drug related and non-drug related deaths. It has been demonstrated that etizolam is a common finding in drug-related deaths in Scotland but there is a lack of data regarding the designer benzodiazepines that emerged after etizolam. This makes toxicological interpretation and the decision to include the drug in the cause of death very difficult for toxicologists and pathologists, respectively, as there is a lack reference ranges to consult. Similarly the scale of use in living populations who are required to abstain from drugs for reason such as treatment or incarceration is unknown. The initial legality and the belief they may evade detection by simple screening tests make the designer benzodiazepines an attractive option.

Two Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) methods were developed in order to test the different sub-populations. The urine method developed

was a qualitative screen and was validated for use. The blood method developed was used to quantify the designer benzodiazepines and was validated for use.

A total of 2,582 samples were analysed from the different sub-populations. Of these, 893 were urine samples from living participants and 1691 were blood samples from deceased individuals. All blood samples were from the post-mortem (PM) cohort and 369 (22%) of the cases were positive for the designer benzodiazepines tested. Diclazepam was detected in 212 cases and gave a median concentration of 0.017 mg/L (n=157, 0.005 – 0.211 mg/L), Delorazepam was detected in 339 cases and gave a median concentration of 0.043 mg/L (n=311, 0.005 – 1.50 mg/L), Lormetazepam was detected in 144 cases and gave a median concentration of 0.010 mg/L (n=85, 0.005 – 0.18 mg/L), Flubromazepam was detected in 18 cases and gave a median concentration of 0.66 mg/L (n=15, 0.01 – 2.30 mg/L), Pyrazolam was detected in 9 cases and gave a median concentration of 0.033 mg/L (n=6, 0.008 – 1.10 mg/L). These concentrations can assist in the toxicological interpretation of these drugs.

The urine samples, which were screened for a wider range of benzodiazepines, were from three different cohorts. These were made up of individuals being admitted to or liberated from one of the seven Scottish Prison Service (SPS) facilities included in this study, individuals under the supervision of a Drug Treatment and Testing Order (DTTO) through the Scottish Drug Court (SDC) system in Glasgow and patients undergoing psychiatric treatment from NHS Greater Glasgow and Clyde Forensic Directorate (NHS GGC FD). The analysis found that 55% of the 73 urine samples from the SDC were positive, 41% of the 725 SPS urine samples were positive and there were no positive samples found in the 95 NHS GGC FD urine samples.

The results of the studies show that benzodiazepines and designer benzodiazepines are widely used in the Scottish population. The individuals from the SDC and NHS GGC FD were able to refuse to take part in the study or able to abstain before their known drug test date. The SPS samples only gives a snapshot of those being admitted to or liberated from prison, which is not a reflection of the general inmate population and not every post-mortem case was tested for designer benzodiazepines. However despite these limitations, key information about the scale, nature and blood

concentrations of the designer benzodiazepines being abused in Scotland was gained.

1. Introduction to Benzodiazepines and Designer Benzodiazepines

1.1. Introduction

The use of benzodiazepines has been a long-term problem in Scotland. In the 1980s, temazepam was the most widely abused prescription drug in the UK (Ashton, 2002) and in 1990s Scotland, there was a brief trend of removing it from its gel capsule to combine with other drugs for injection. Scottish drug users are known to take “supratherapeutic” or mega doses of benzodiazepines as it can increase the high from heroin and increase intoxication when used together. (Johnson, Barnsdale and McAuley, 2016) An audit of drug prescribing in NHS Greater Glasgow and Clyde in 2013 found that 25% of opioid users were prescribed one or more benzodiazepine type drugs. (Johnson, Barnsdale and McAuley, 2016) It seems that Scottish drug users have always sought out the use of benzodiazepines, therefore when a wave of novel benzodiazepines were legally for sale in Scotland from 2010 onwards, these were an attractive addition to the drug using population. Some of these drugs were completely new preparations, others had been patented in the 1960s and 70s but never fully investigated or brought to market. In the years that followed the Scottish drug landscape changed rapidly with the availability of new ‘legal’ drugs. These changes presented challenges for not just the users and their families but the treatment services, policymakers and the laboratories that were responsible for detecting these drugs.

This work will investigate the use of some of these designer benzodiazepines in blood from deceased individuals and in urine of living participants from a population with a history of drug use, a population under psychiatric treatment and individuals leaving or entering the Scottish prison system. The drugs investigated in post-mortem blood were diclazepam and two of its metabolites (delorazepam and lormetazepam), pyrazolam and flubromazepam. This analysis dealt with active post-mortem cases and was part of the wider toxicological investigation. These drugs were chosen, as there was increasing evidence from various agencies such as the police and online searches that these drugs may be getting abused in Scotland. The urine screen used was more encompassing with 22 analytes included to give a fuller picture.

1.2. History of benzodiazepines

The first benzodiazepine discovered was chlordiazepoxide, which was synthesised accidentally by Dr Leo Sternbach. It was initially placed in storage with no further investigation until it was rediscovered and submitted for pharmacological evaluation during a laboratory clean up in 1957. It was found to have hypnotic, sedative and anti-strychnine effects. The structure was identified as a 1, 4-benzodiazepine and it was introduced into clinical use in 1960. (Ban, 2006; Lader, 1991) Creating new benzodiazepine analogs showed promise that a new, safer alternative to the addictive barbiturates had been discovered. Benzodiazepines appeared to be non-addictive unlike barbiturates as initial trials showed the users were not asking for an increased dose. (Ban, 2006; Lader, 1991)

Diazepam, now the most well-known benzodiazepine was introduced 1963 and became the most popular benzodiazepine worldwide in the 1960s and 1970s. (Mehdi, 2012)

Table 1 shows the introduction of benzodiazepines into the UK pharmaceutical market. Some of the drugs displayed in the table are no longer available on prescription in the UK, alprazolam is an example of this. It is still commonly prescribed in the USA. Ketazolam and flunitrazepam are also no longer prescribed in the UK. Flunitrazepam and especially the brand name Rohypnol have a negative connotation in popular culture as an untraceable ‘date rape’ drug.

Table 1: UK introduction of benzodiazepines

Drug Name	Brand Name	Year introduced
Chlordiazepoxide	Librium	1960
Diazepam	Valium	1963
Nitrazepam	Mogadon	1965
Oxazepam	Serenid	1966
Lorazepam	Ativan	1972
Temazepam	Euhypnos	1977
Clobazam	Frisium	1979
Ketazolam	Anxon	1980
Lormetazepam	Noctamid	1981
Flunitrazepam	Rohypnol	1982
Alprazolam	Xanax	1983

Table amended from Lader 1991 (Ban, 2006; Lader, 1991)

1.2.1. The emergence of designer benzodiazepines

The term New (or Novel) Psychoactive Substances (NPS) is defined by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) as ‘a new narcotic or psychotropic drug, in pure form or in preparation, that is not controlled by the United Nations drug conventions, but which may pose a public health threat comparable to that posed by substances listed in these conventions'. (EMCCDA) NPS are often called designer drugs and are usually, but not exclusively, structural analogues designed to mimic the effect of a traditional drug of abuse while circumventing the legislation. (Wohlfarth and Weinmann, 2010; Stephenson and Richardson, 2014)

In 2007, the EMCDDA expanded its early warning system (EWS) scope to include all NPS; this saw a dramatic increase in compounds reported to the EMCCDA each year.

According to the EMCDDA, phenazepam was the first designer benzodiazepine to hit the recreational market in 2007 and from around 2012 phenazepam and etizolam were sold by online retailers as ‘research chemicals,’ (EMCDDA., 2015) See figure 1 for a timeline of designer benzodiazepines and when they were reported to the EMCDDA.

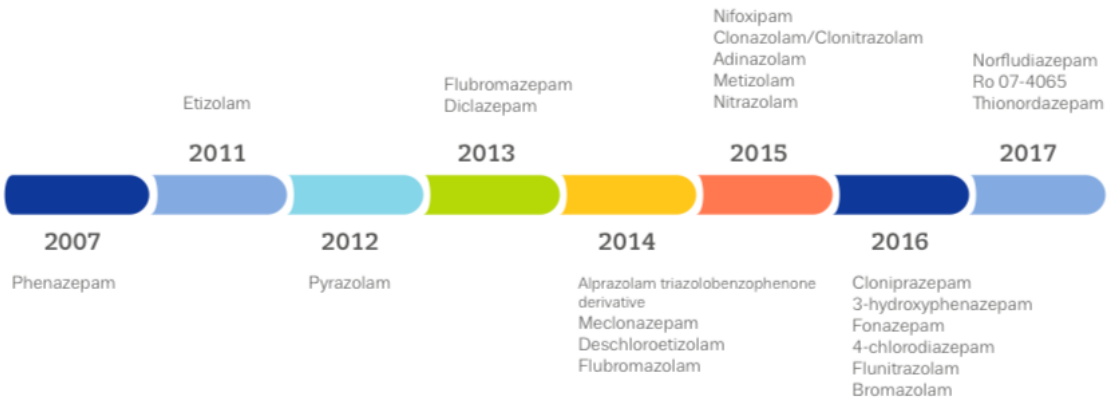

Figure 1: EMCDDA timeline of reported designer benzodiazepines. (EMCDDA, 2015)

From around 2012/2013, stores in city centres across Britain, known as ‘head shops’, would also stock these research chemicals. Stimulants and synthetic cannabinoids were available alongside the designer benzodiazepines in these shops and online. Figure 2 shows two packets of designer benzodiazepines purchased in a shop in Edinburgh in November 2014. The packaging of the drugs

states ‘Not for human consumption’ which at the time was a way to avoid providing any information about the use of the drug or the harms and circumvent trading standards legislation. (Stephenson and Richardson, 2014) A report in 2016 placed the UK as the second highest country (after the USA) of dark net vendors selling illegal drugs. (Kruithof, 2016) Etizolam, diclazepam, flubromazepam, phenazepam and pyrazolam are the most frequent designer benzodiazepines reported to the EMCDDA. Etizolam, diclazepam, flubromazepam and phenazepam make up 80% of the European drug seizures of designer benzodiazepines since 2005.

Figure 2: Designer benzodiazepine packets purchased in a head shop in Edinburgh in 2014

Designer benzodiazepines have existed for decades before they became a drug sold for recreational purposes, Table 2 shows the year each designer benzodiazepine was first patented and the year it was reported to the EMCDDA EWS. Due to this, the term NPS should be thought of as newly misused or newly available on the drug market as these substances are often not inherently new, some are prescribed in other countries. The availability of these drugs was concerning for drug treatment services, the NHS and toxicology laboratories. These drugs were potentially evading their detection and causing unknown harms to the drug using population.

Table 2: Designer benzodiazepine patent date and date reported to EMCDDA

Drug	Year patented	Year reported to EMCDDA
Phenazepam	1974	2007
Etizolam	1978	2011
Pyrazolam	1979	2012
Diclazepam	1964	2013
Flubromazepam	1962	2013
Nifoxipam	1985	2014
Flubromazolam	1978	2014
Clonazolam	1971	2014
Deschloroetziolam	1998	2014
Metizolam	1988	2015

Table amended from Manchester *et al* (Manchester *et al.*, 2018)

Google trends is a service which analyses the popularity of Google search terms in a geographical location and in any selected period of time. Figure 3 shows the trends for phenazepam, etizolam, diclazepam and flubromazepam from the beginning of 2012 to the beginning of 2019 in Scotland. Etizolam is the most popular drug searched and its popularity peaked in September 2014. February 2012 was the peak of phenazepam searches, diclazepams' search peak was August 2015 and flubromazepams' was October 2014. Most of the other designer benzodiazepine had very little data on Google trends so have not been included.

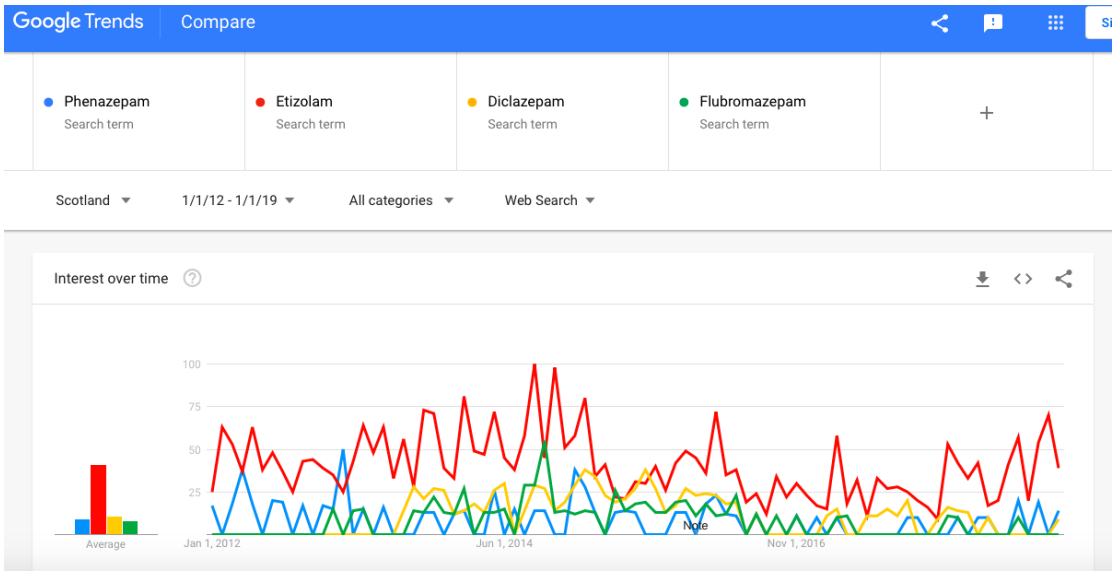

Figure 3: Google trends data showing the search popularity of four designer benzodiazepines over a 7-year period

1.3. Prescribing benzodiazepines

Benzodiazepines have been used effectively for many clinical reasons such as anxiety, depression, stress, withdrawal therapy from drugs and alcohol, treatment of seizures, treatment of sleep disorders, palliative care, pre/post-operative care and as muscle relaxants. (Mehdi, 2012) Benzodiazepines are grouped into short, medium and long acting characterised by the half-life of the parent drug. A long-acting benzodiazepine such diazepam has a half-life of 20 to 50 hours, a medium acting such as lorazepam has a half-life of 10 to 20 hours and a short acting benzodiazepine such as midazolam a half-life of 1 to 4 hours. The duration of action determines their clinical use. The long to medium acting drugs are useful in treatment of anxiety, as this tends to be a reoccurring condition. The short acting benzodiazepines are used in anaesthesia and palliative care, as they are not required for a lengthy time.

The apparent safe nature and their usefulness in treating multiple conditions led to a huge rise in prescribing benzodiazepines; in 1980 around 80 billion doses of benzodiazepines were consumed each day worldwide. (Tyrer, 1980) In April 2013, NHS Greater Glasgow and Clyde issued guidance to ban prescribing 10 mg diazepam doses (known as “blue vallies”) and recommended 2 mg instead, This was also a way to determine fake “blue vallies” from prescription ones however yellow or white fakes were also then produced. Users in Glasgow often take many tablets at the one time and a lower dose is therefore safer. (NHS Greater Glasgow & Clyde Area Drug and Therapeutics Committee, 2013) This reinforces the high abuse potential of benzodiazepines in Glasgow and the attempts local authorities have put in place to try and curb that abuse.

Benzodiazepines are useful for treating conditions such as mild anxiety however prescribing them to individuals with severe mental illnesses such as schizophrenia and bipolar disorder can exacerbate the symptoms such as anxiety and depression. A study found that individuals with a mental health disorder and a substance abuse history are unlikely to refrain from drug abuse when prescribed benzodiazepines and are likely to abuse them. The study was made up of 203 patients who were assessed through yearly follow up interviews over six years, 43% of participtants were prescribed benzodiazepines at the time of at least one assessment. Relying on interviews can lead to under reporting and as they were conducted yearly, intermittent benzodiazepine use may have been missed

nevertheless alternative approaches to managing symptoms should be sought for these individuals. (Brunette *et al.*, 2003) The mismanagement and over-prescribing of benzodiazepines by health care professionals within acute mental health settings is also reportedly a concern. This can lead to tolerance, addiction and can be dangerous when used in combination with other CNS depressants. (Duxbury and Baker, 2004)

The benzodiazepines, diazepam, temazepam, oxazepam, nitrazepam, lorazepam, lormetazepam, loprazolam and chlordiazepoxide were all dispensed in Scotland in 2017/18. (Information Services Division Scotland, 2018) Diazepam was the most commonly dispensed (879,177 times) and lormetazepam the least dispensed (2,533 times) over the year period. Figure 4 shows how many times each benzodiazepine was dispensed in Scotland in 2017/2018. Whilst this doesn't give information on how many people received these drugs, it gives some scale to how many legitimate prescriptions of these drugs there were.

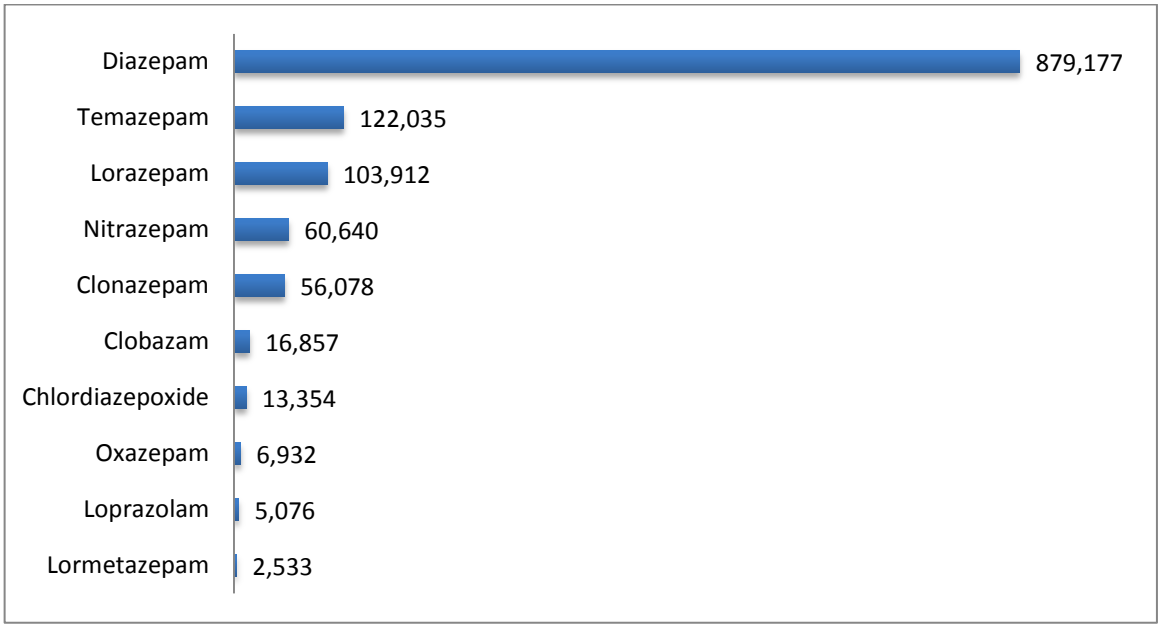

Figure 4:Number of benzodiazepines dispensed in Scotland in 2017/2018

1.4. Abuse of benzodiazepines

Opioid users frequently also misuse benzodiazepines; this can be to increase the high from the opioid(s) or to self-medicate to treat withdrawals or psychological issues. It has been shown in the ample evidence summarised in the review by Jones *et al* how opioids and benzodiazepines are commonly co-abused and that

the intensity and duration of opioid drugs can be prolonged by the concomitant use of benzodiazepines, (Jones, Mogali and Comer, 2012) studies have especially focused on benzodiazepine use in those on opioid replacement therapy. Methadone users reported stronger highs and euphoria when used with diazepam however this was a small study of only five patients (Spiga *et al.*, 2001) Greater and prolonged respiratory depression was observed when midazolam was given intravenously with buprenorphine then one drug alone in rodents, this was determined by sampling the arterial gases and pH. (Gueye *et al.*, 2002) This indicates injecting benzodiazepines along with opioids may be particularly dangerous however oral administration is the most common way to consume benzodiazepines. (EMCDDA., 2015)

Intelligence on the drugs being seized by the police on the street can give an indication on what drugs are being abused. This can be a very valuable resource in dynamic drug markets as it provides a relevant snapshot and often informs what drugs forensic laboratories should analyse for. The number of any type of drugs seized from year to year can vary dramatically; in 2016/2017 2.2 million benzodiazepine tablets were seized in Scotland by police. (Scottish Government., 2019) In 2017/2018 Police Scotland seized 321,000 diazepam or other benzodiazepine tablets, this number does not include etizolam. The number of etizolam tablets seized was 240,000. Figure 5 shows the percentage of drug seizures in 2017/2018 in Scotland, this data demonstrates that benzodiazepine tablets are a common drug to find on Scottish streets, the presence of benzodiazepines that are not diazepam on the street may be the more significant information rather than the absolute number of seized drugs.

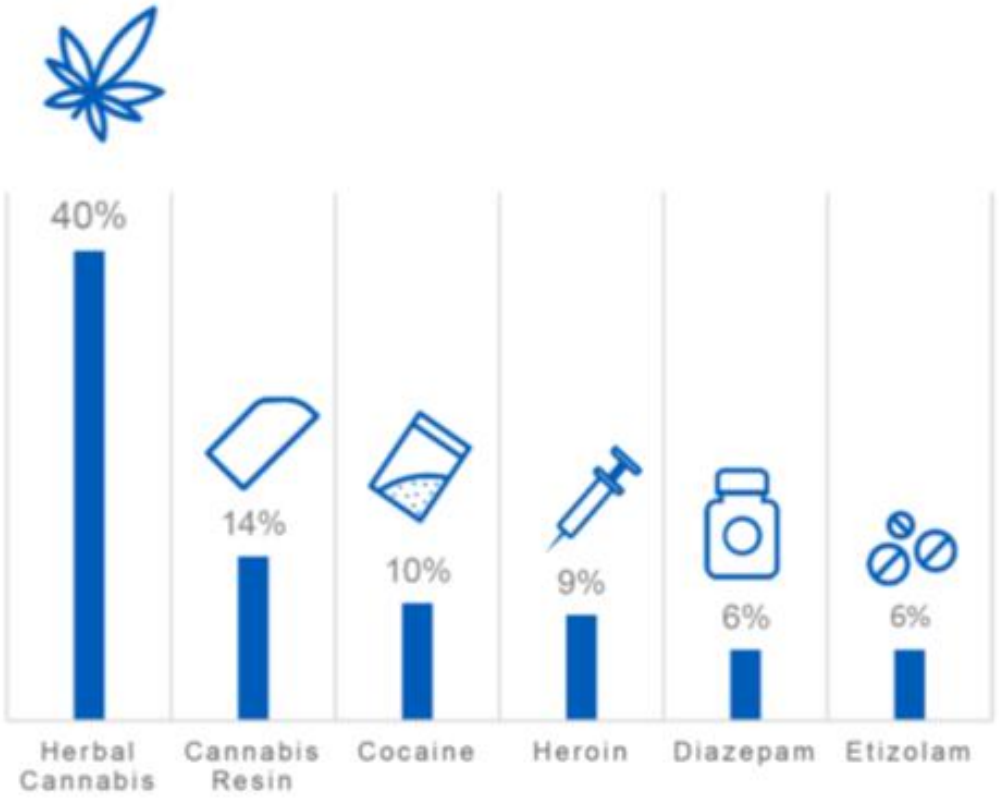

Figure 5:Percentage of drugs including diazepam and etizolam in Police Scotland drug seizures 2017/2018

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Another way to gauge the extent of benzodiazepine use or abuse is to assess its prevalence and how it compares to the other drugs detected in driving populations. One study investigating the post-mortem blood results of drivers in fatal collisions from 2012 to 2015 in Scotland found that benzodiazepines was the third most common drug group found after cannabis and opioids. This benzodiazepine finding was in 12% of the cases examined (n=14 out of the 118 cases examined). Diazepam was found in 13 out of 14 cases; once in combination with phenazepam (a designer benzodiazepine) and in one case phenazepam was found on its own. Due to benzodiazepines being a common drug of abuse in Scotland and a lack of prescription information for diazepam, it was not clear if these cases were from illicit or therapeutic use. (Hamnett *et al.*, 2017) Another driving study in Scotland noted a dramatic increase in arrested drivers who tested positive for benzodiazepines from the time period of 1996 to 2000 (39%) and in 2008 (85%). This study utilised a screening method only and the positive results were not

confirmed. (Officer, 2009) Screening tests are further described in Chapter 2, screening tests are non-specific tests and therefore the use of a screening method alone means that there is a chance of false positives and the results of the study may not be accurate. The study does not mention any change in the screening method over time and therefore the false positive rate could be consistent leading to comparable results and a valid conclusion of an increase in benzodiazepine use in drivers in Scotland from 2000 to 2008.

1.5. Structure

Understanding of molecular structure of drugs is essential in forensic toxicology in order to make decisions on how best they can be analysed, the similarity or differences between drugs can be utilised for the identification of the compound.

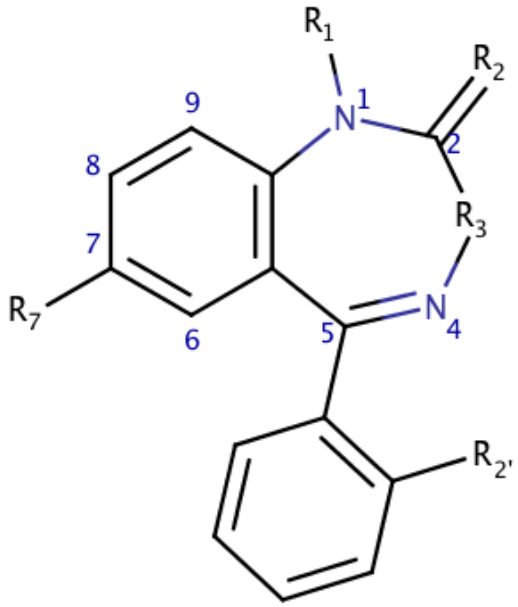

Figure 6: The structure of a 1, 4-benzodiazepine

The general structure of a 1,4 benzodiazepine is made up of a seven-member diazepine ring with a fused benzene ring and a phenyl ring, see Figure 6. Table 3 shows the differences in the structural positions of common benzodiazepines.

Table 3: The structural positions of common benzodiazepines

Drug	R_1	R_2	R_3	$R_{2'}$	R_7
Diazepam	CH_3	O	H_2	H	Cl
Desmethyldiazepam	H	O	H_2	H	Cl
Temazepam	CH_3	O	OH	H	Cl
Oxazepam	H	O	OH	H	Cl
Lorazepam	H	O	OH	Cl	Cl

1.5.1. Designer benzodiazepines structure

Designer benzodiazepines have a similar structure to the commonly prescribed benzodiazepines, for example an additional chlorine atom is the only difference between diclazepam and diazepam. The addition of the chlorine makes diclazepam a more potent drug than diazepam. The addition of a halogen such as fluorine or chlorine at the R2' position results in significantly enhanced activity at the receptor level. (Moosmann and Auwärter, 2018) Table 4 and Figure 7 show the differences in the structural positions of designer benzodiazepines.

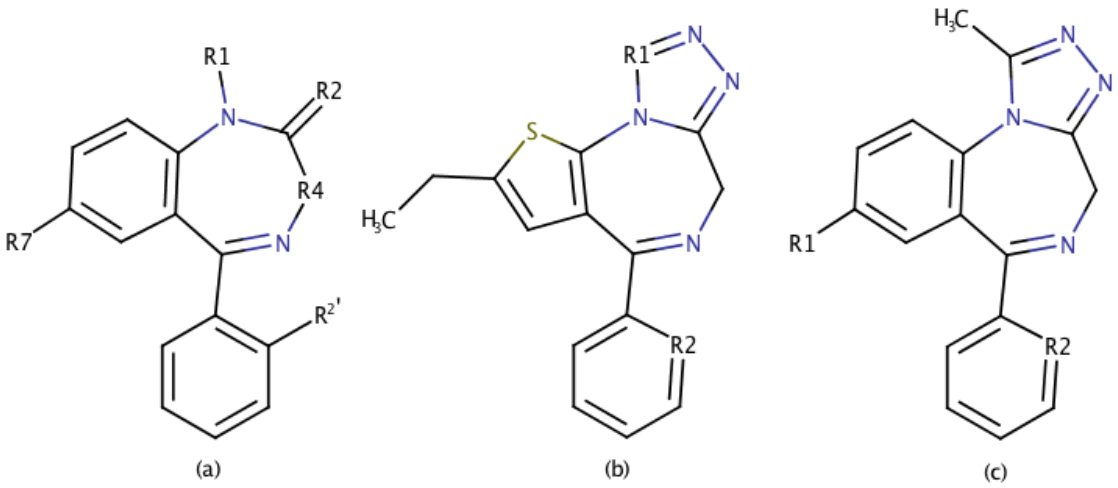

Figure 7: The structure of a 1,4 benzodiazepine (a), thienotriazolodiazepine (b) and triazolodiazepine (c)

Table 4: The structural positions of designer benzodiazepines

Drug	R1	R2	R2'	R7
1, 4 benzodiazepines				
Diclazepam	CH_3	O	Cl	Cl
Flubromazepam	H	O	F	Br
Meclonazepam	H	O	Cl	NO_2
Nifoxipam	H	O	F	NO_2
Phenazepam	H	O	Cl	Br
Thienotriazolodiazepines				
Deschloroetizolam	CH_3	H	-	-
Etizolam	CH_3	Cl	-	-
Metizolam	H	Cl	-	-
Triazolodiazepine				
Clonazolam	NO_2	Cl	-	-
Flubromazolam	Br	F	-	-
Pyrazolam	Br	H	-	-

There does not appear to be a relationship between structure and half-life therefore it is not possible to predict the half-life based on another benzodiazepine, for example diclazepam and flubromazepam share the 1,4 benzodiazepine structure and have only slight differences at positions R1, R2 and R5 however diclazepam has a much shorter half-life of 46 hours compared to flubromazepam estimated half-life of 106 hours. This increases the potential hazard for recreational users consuming benzodiazepines that there is little information about. Inadvertent re-dosing with a compound that has a very long half-life could result in toxicity due to unintended drug accumulation. (Greenblatt and Greenblatt, 2019) A study from 2018 developed a model to predict the receptor binding affinity of the new benzodiazepines, the model known as quantitative structure-activity

relationship (QSAR) postulated the benzodiazepines classified under the Psychoactive Substances Act (PSA) did have a greater binding affinity than the medically prescribed benzodiazepines, the analysis concluded the triazolobenzodiazepines were the most potent group. (Waters *et al.*, 2018) However, there are no *in vivo* studies to test this theory. Benzodiazepines can be classified by their structure, their duration of action or their half-life. Designer benzodiazepines are best classified by their structure as this information is fully known. Etizolam, phenazepam and alprazolam are not technically designer benzodiazepines as they are prescribed in other countries; etizolam is used medicinally in Japan, phenazepam in Russia and alprazolam in the USA. However they are not prescribed in the UK and their use in Scottish samples indicates illicit benzodiazepine use, therefore for simplicity they will be considered designer benzodiazepines in this study.

Figure 8 shows the full structure of the diclazepam, pyrazolam and flubromazepam, the three drugs of particular interest in this research, predominantly in post-mortem blood.

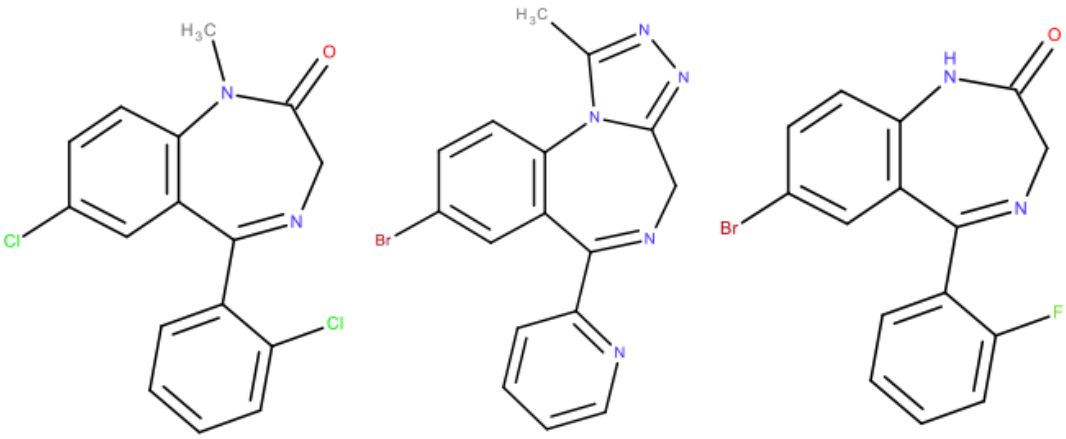

Figure 8: Diclazepam, pyrazolam and flubromazepam structures

1.6. Mechanism of action

The mechanism of action explains on a cellular level how the drug has an effect on the body. In order to understand how benzodiazepines work on the human body knowledge of their mechanism of action is required, therefore it is quite astonishing that the benzodiazepine receptor was not detailed in literature until 1977, 17 years after diazepam was first prescribed. (Möhler and Okada, 1977) It is now understood that benzodiazepines bind allosterically to the gamma-aminobutyric acid A (GABAA) receptors in the central nervous system (CNS). GABA is the major inhibitory neurotransmitter in the CNS. The GABAA receptor is a protein complex, which is made up of five subunits forming a chlorine ion channel. There are 16 subunits within the seven subunit families (α 1-6, β 1-3 γ 1-3, δ, ε, π, θ) that make up various combinations; the most common combination in the brain is α2β2γ. (Olsen and Sieghart, 2009) Benzodiazepines bind allosterically between the α1 and γ2 subunits. Allosteric binding describes the binding of a molecule to the receptor at a site other than the neurotransmitter site; therefore the molecule has an indirect effect. Figure 9 shows a schematic diagram of the receptor; the A denotes the benzodiazepine binding site and the B denotes the GABA binding site. Benzodiazepine binding increases the frequency of the opening of the chlorine channel when neurotransmitter gamma-aminobutyric acid (GABA) is bound to its own site on the receptor. An increased concentration of chlorine ions in the postsynaptic neurons results in a hyperpolarised membrane, this gives a sedative effect, as the membrane is less excitable. (Sieghart, 1995) Ethanol and barbiturates are examples of other drugs which also bind allosterically to the GABAA receptor. Barbiturates work in a slightly different way at the receptor level than benzodiazepines, they do not need GABA to also be bound to the receptor and they increase the length of time the ion channel is open rather than the frequency. As a result, barbiturates are capable of greater CNS depression and toxicity than benzodiazepines. (Tan, Rudolph and Lüscher, 2011)

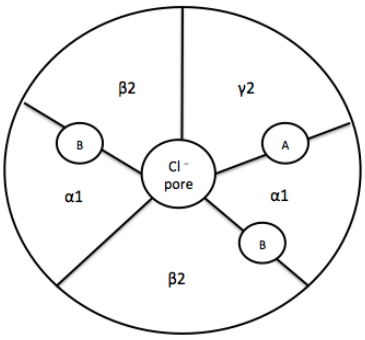

Figure 9: The $GABA_A$ receptor protein complex

The potential for benzodiazepine dependence is still not fully understood, there are studies that have suggested dependence is purely psychological and is associated with the ritual of daily benzodiazepine use. (de las Cuevas, Sanz and de la Fuente, 2003) However other studies have suggested there is potential for addiction as withdrawal symptoms such as tremors are often noted after the cessation of use. These symptoms are likely from neuroadaptions attempting to maintain the body's homeostasis by overcoming the increased depression of the CNS. When the use of benzodiazepines is halted these neuroadaptions cause the sudden hyper-excitability of the nervous system and this is the appearance of withdrawal symptoms. (Allison and Pratt, 2003)

1.7. Metabolism

Drug metabolism is extremely valuable for toxicologists as the metabolites provide markers for drug detection. Benzodiazepine metabolism in particular can be complex as many parent drugs can produce the same metabolites in the body and some will metabolise to drugs that are also prescribed e.g temazepam is a parent drug and a metabolite of diazepam making interpretation complex. Blood concentrations are required to assess the ratio of the drugs to determine what may have been ingested.

Metabolism is the chemical alteration of a drug by the body for the purpose of excretion. It consists of two stages known as phase I and phase II metabolism. Phase I is responsible for the transformation or addition of a functional group by oxidation, reduction or hydrolysis. Phase II is responsible for conjugation, this transforms the molecule by conjugating the drug or metabolite to an endogenous molecule such as glucuronic acid. (J.-P. Tillement, 2007) Oxidation and

glucuronidation are the two main pathways in benzodiazepine metabolism. (Mandrioli, Mercolini and Raggi, 2008)

The phase I oxidation step is carried out by cytochrome P450 which is a superfamily of enzymes. The main cytochrome P450 enzymes involved in diazepam metabolism are CYP3A4 and CYP2C19. CYP3A4 is the most common isoform found in the human liver and is responsible for catalysing the metabolism of over 50% of clinical drugs. (Mizuno *et al.*, 2009) Genetic polymorphisms (individual differences determined by genetics) in the cytochrome P450 enzymes cause variation in how individuals metabolise a drug (e.g. fast and slow metabolisers). (Gibson and Skett, 2013; J.-P. Tillement, 2007) The oxidation of benzodiazepines by the cytochrome P450 system produces active metabolites, which in turn have to be glucuronidated to be excreted. The duration of this process and the production of active metabolites mean the duration of action can be very long. Drugs such as oxazepam and lorazepam that are metabolised by direct glucuronidation have short durations of action as the glucuronidation produces inactive metabolites that are rapidly excreted. (Mandrioli, Mercolini and Raggi, 2008) Figure 10 shows how diazepam is metabolised to desmethyldiazepam and temazepam, which are then both metabolised to oxazepam, which is then conjugated into a glucuronide metabolite for excretion. Temazepam is also excreted by glucuronidation. Diazepam and desmethyldiazepam are the main analytes detected in the blood as oxazepam and temazepam undergo glucuronidation and are excreted at almost the same rate as they are produced. (Mandrioli, Mercolini and Raggi, 2008)

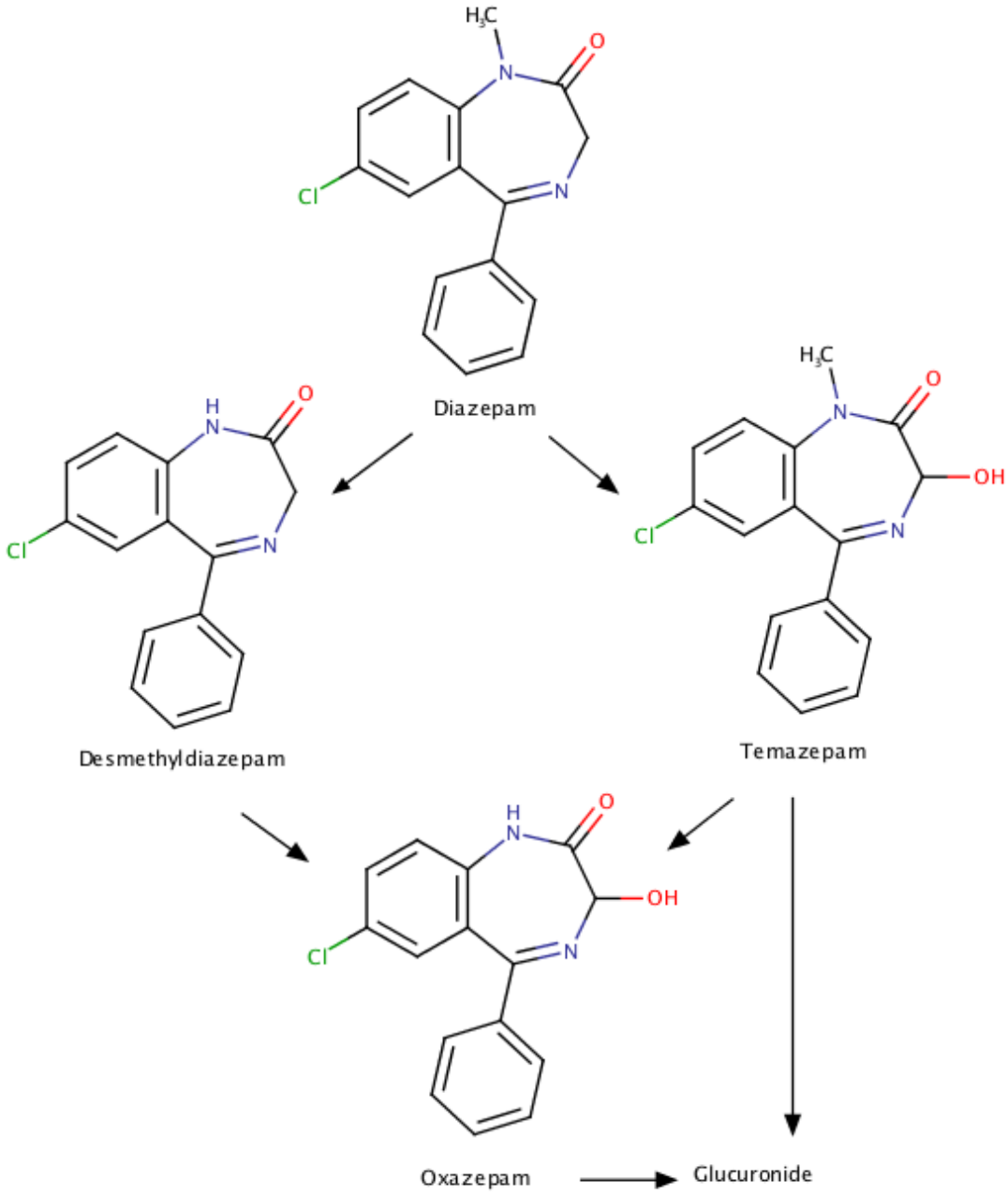

Figure 10: Diazepam metabolism

1.7.1. Designer benzodiazepines metabolism

As designer benzodiazepines have not been subjected to clinical trials the way pharmaceutical benzodiazepines have, there is limited information on their metabolism and pharmacokinetic properties. However recent studies have shown they undergo the same pathways of oxidation and glucuronidation as pharmaceutical benzodiazepines. (Moosmann, Bisel and Auwärter, 2014; Moosmann and Auwärter, 2018) Figure 11 shows how diclazepam is metabolised to delorazepam and lormetazepam, which are then both metabolised to

lorazepam, which is turned into a glucuronide for excretion. Lormetazepam and delorazapam are also excreted by glucuronidation.

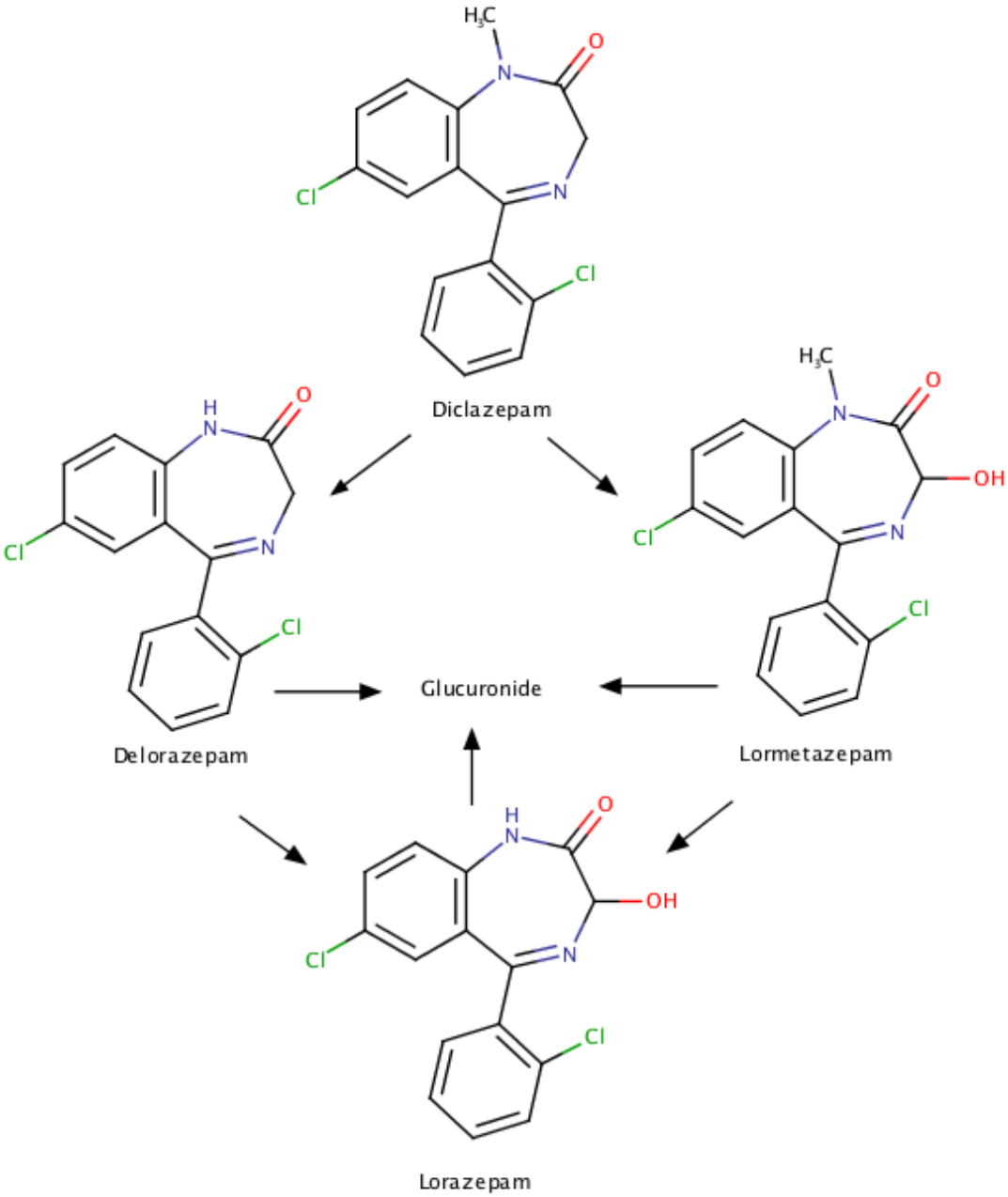

Figure 11: Diclazepam metabolism

A study by Moosmann *et al* identified two hydroxyl metabolites for flubromazepam, (Moosmann *et al.*, 2013a) one of these metabolites was debrominated which is thought to be the result of bacterial degradation of flubromazepam in the gut. (Moosmann and Auwärter, 2018) The main metabolite is 3-hydroxyflubromazepam. (Moosmann and Auwärter, 2018) Both hydroxyl

metabolites are glucuronidated and only a low concentration of the parent drug was detected in urine. Figure 12 shows the metabolism of flubromazepam. (Moosmann *et al.*, 2013a)

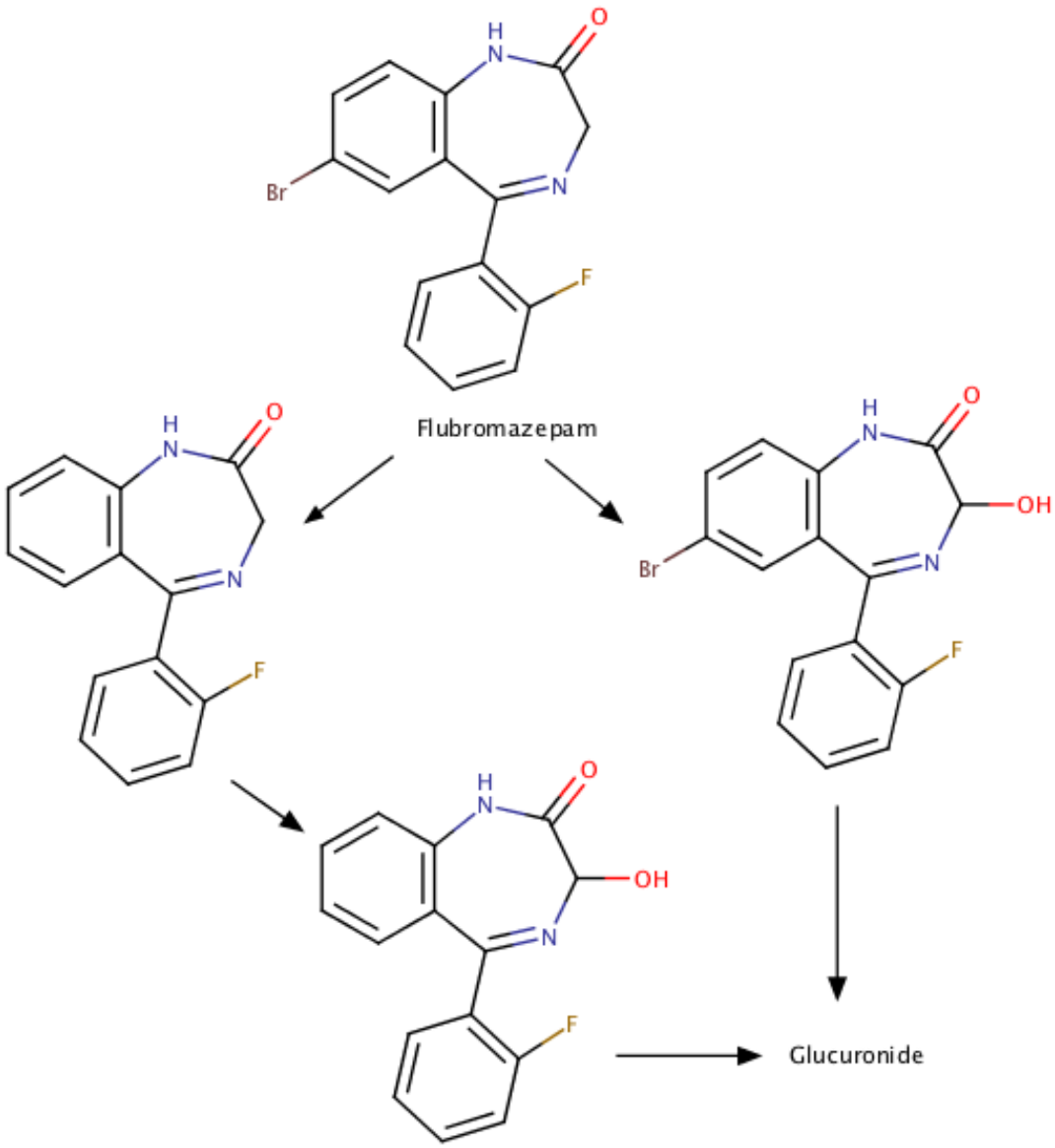

Figure 12: Flubromazepam metabolism

Initial studies suggested that pyrazolam was not extensively metabolised, as the unchanged parent drug can be easily detected in urine. (Moosmann *et al.*, 2013b) However a subsequent study has shown a parent glucuronide as well as a hydroxyl glucuronide. (Pettersson Bergstrand *et al.*, 2018)

The elimination half-life of some designer benzodiazepines has been estimated using volunteer studies. (Moosmann, Bisel and Auwärter, 2014; Moosmann *et al.*,

2013b; Moosmann and Auwärter, 2018; Moosmann *et al.*, 2013a; Huppertz *et al.*, 2015) Self-administration of a low dose of some designer benzodiazepines in serum by healthy volunteers was described in articles by Moosmann *et al* and Huppertz *et al*, Table 5 gives a summary of the dose and half-life. (Moosmann *et al.*, 2013b; Moosmann *et al.*, 2013a; Moosmann, Bisel and Auwärter, 2014; Huppertz *et al.*, 2015) As these studies are one instance of the drug being ingested once by one person it is very preliminary data.

Table 5: Results from four designer benzodiazepine self-administration studies

Drug	Dose ingested (mg)	Maximum concentration reached (mg/L)	Elimination half-life (h)
Pyrazolam	1	0.051	17
Flubromazepam	4	0.078	106
Diclazepam	1	0.003	42
Flubromazolam	0.5	0.009	10-20

The metabolism of phenazepam is not well documented in literature and this poses an issue as 3-hydroxyphenazepam was reported to the EMCDDA in 2016 as a designer benzodiazepine however it is also a phenazepam metabolite. One study demonstrated how 3-hydroxyphenazepam only fortified samples contained phenazepam; this is thought to be due to thermal instability. This makes interpretation complex, as it is not clear what has been consumed. (Crichton *et al.*, 2015)

1.8. Legislation

All benzodiazepines are Schedule IV controlled drugs, with the exception of flunitrazepam, which is a Schedule III controlled drug under the UN Convention on Psychotropic Substances. This is an international treaty designed to control psychoactive drugs. (United Nations Office Drugs and Crime, 1968) Benzodiazepines are controlled under the Misuse of Drugs Act 1971 in the UK, as Class C drugs. (*Misuse of Drugs Act 1971*, 2019) Class C drugs are considered to be the drugs, which have the least capacity for harm of all controlled drugs, however it has been suggested that the classification does not accurately reflect potential for harm. (Nutt, King and Phillips, 2010) The benzodiazepines that are medicines in the UK, such as diazepam and lorazepam are listed under schedule 4 part 1 of the Misuse of Drugs Regulations 2001. The Misuse of Drugs

Regulations 2001 determines the legitimate use for controlled drugs. Schedule 4 part 1 prohibits the production of the drugs listed but allows for medicinal consumption. (*The Misuse of Drug Regulations 2001*, 2001)

An amendment to The Misuse of Drugs Act 1971 in May 2017 classified most designer benzodiazepines as a Class C drug in the UK. (*Misuse of Drugs Act 1971*, 2019) The designer benzodiazepines are listed under schedule 1 the Misuse of Drugs Regulations 2001. Schedule 1 lists the drugs that have no medicinal use in the UK. (*The Misuse of Drug Regulations 2001*, 2001) The Advisory Council on the Misuse of Drugs (ACMD) recently released a report recommending the classification of a 13 further designer benzodiazepines such as flualprazolam as Class C drugs under the Misuse of Drugs Act 1971 and as Schedule 1 under the Misuse of Drugs Regulations in line with the other designer benzodiazepines. (Advisory Council on the Misuse of Drugs, 2020)

1.9. Benzodiazepines in drug related deaths

Collating the data for drugs which are present in deaths and in particular drug related deaths (DRD) allows trends to be identified in drug use and more importantly can inform which drugs are most dangerous or which concentrations of drugs are dangerous. Benzodiazepines are not known to be fatal drugs however their presence amongst drug deaths is common. This is due to them being used in combination with of other drugs particularly opiates as discussed in section 1. 4.

The Office for National Statistics report on 'Deaths related to drug poisoning in England and Wales: 2018 registrations' showed around 10% of drug poisoning deaths included the name of a benzodiazepine in the cause of death. In the report the definition of a drug related death is given as "a …death …where either the underlying cause is drug abuse or drug dependence, or the underlying cause is drug poisoning and any of the substances controlled under the Misuse of Drugs Act 1971 are involved." (Office for National Statistics, 2019)

Figure 13 shows how many cases mentioned a benzodiazepine in the cause of death in England and Wales from 1993 to 2018. In recent years there has been an upward trend however there has been an increase in drug-related deaths since 2012. Previously these deaths had mostly been linked to heroin/morphine however the 2018 dataset included a wider variety of drugs including cocaine and more "novel" drugs. (Office for National Statistics, 2019)

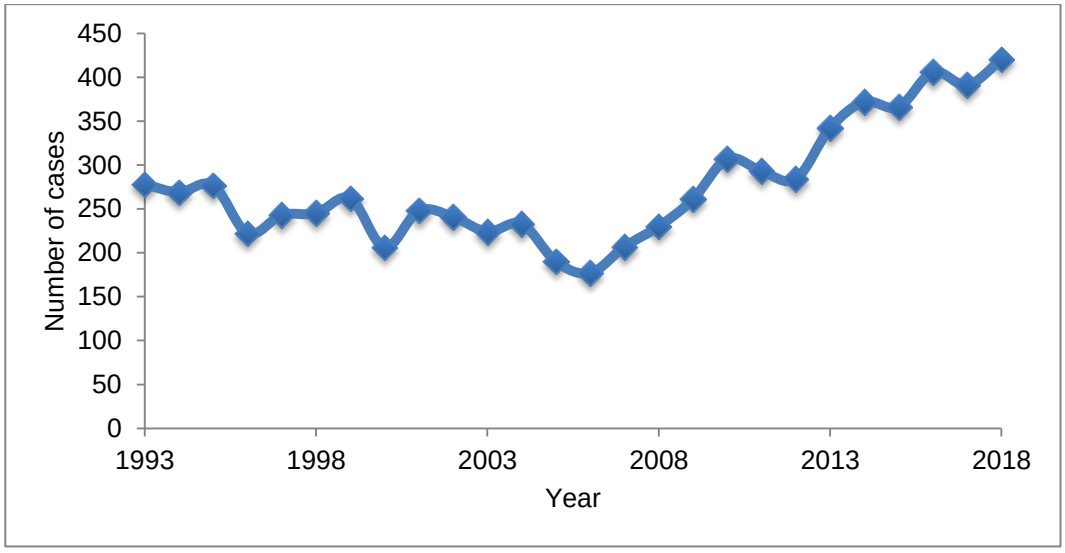

Figure 13: Number of cases with a benzodiazepine mentioned in the cause of death in England and Wales over a 25 year period
(Office for National Statistics, 2019)

The National Records for Scotland "Drug-related deaths in Scotland in 2018" report found that 2018 had the highest ever number of drug deaths at 1,187; a 27% increase from the 2017 figures. The report states they use the same definition as the Office of National Statistics for a drug related death. The report also determined that 72% of people dying of a drug related death were male and the two age groups spanning 25-44 years old had the largest number of drug related deaths. Benzodiazepines were implicated or potentially contributed to 792 drug related deaths, which is 67% overall. The report states that 'street' benzodiazepines such as etizolam were implicated or potentially contributed to 765 drug related deaths, which is 57% overall. 'Prescribable' benzodiazepines such as diazepam are noted to be implicated much less; 228 deaths or 20% overall. There were 11 deaths that were attributed to one benzodiazepine only with no other drugs detected other than alcohol in some cases; 7 of these 11 were due to etizolam. Figure 14 shows the implication of benzodiazepines in drug related deaths in Scotland from 2000 to 2018 and the increase in recent years. Figure 14 also displays the percentage of drug related death cases where a benzodiazepine has been included; this shows the increasing trend since 2014.

The percentage of benzodiazepines included in the cause of death in Scotland has been under 40% from 2004 to 2014, it has been relatively stable for these 10 years. Perhaps this is partly due to pathologists recognising the traditional benzodiazepines such as diazepam, when new drugs appear however this leads

to more uncertainty and they are hesitant to exclude them from the cause of death. The increase may also be simply due to the rise in popularity of benzodiazepine use in drug users. Interestingly, a study published in 2004 compared the concentrations of free morphine in the post-mortem blood with co-existant drugs and concluded that benzodiazepines result in a higher free morphine concentration compared to morphine and alcohol alone. This suggests that benzodiazepines are playing an important role in drug-related drugs in particular however the cause of the high concentration is not clear and may be a result of pharmacokinetics, user tolerance or user error due to intoxication. (Stenhouse, Stephen and Grieve, 2004)

Comparison of Figure 13 and 14 demonstrates how Scotland has a much bigger relationship with benzodiazepine use compared to England and Wales. Scotland's drug deaths (per head of population) are almost three times larger than that of the UK as a whole. (National Records of Scotland, 2019) However it is not clear if all areas are comparable, it may be that some regions test for a reduced benzodiazepine panel in drug related deaths and may not test for the likes of etizolam and other designer benzodiazepines. Scotland may detect more benzodiazepines in drug related deaths due consistent testing, as these drugs have been popular in the country for decades.

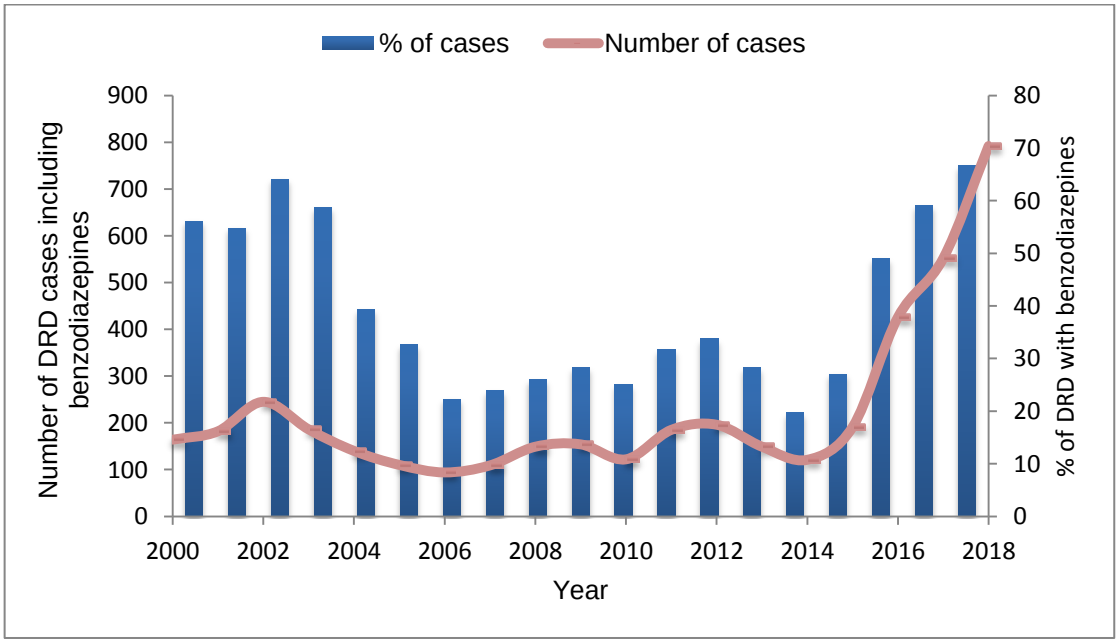

Figure 14: Number of cases with benzodiazepine reported in a drug-related death in Scotland over a 19 year period.

1.9.1. Designer benzodiazepines role in drug related deaths

There were 575 deaths in which NPS were implicated in 2018 in Scotland; designer benzodiazepines were the only NPS present in 571 of those. (National Records of Scotland, 2019) Etizolam was the most implicated designer benzodiazepine in drug-related deaths and NPS-related deaths although the report does not specify the exact etizolam figure for the NPS deaths. Other designer benzodiazepines mentioned were alprazolam (alprazolam is a prescribed drug in other countries such as the USA, however it is used illicitly in the UK), which was included in 137 drug-related deaths then diclazepam in 31 and phenazepam in 27. It is worth noting that the report states if a death has a medical condition selected first as the cause of death then a drug will not be counted in the statistics, despite being named. An example given to explain this would be a cause of death given as 'Coronary artery thrombosis and morphine, etizolam and diclazepam intoxication' - this would not be counted as a drug related death. (National Records of Scotland, 2019) Therefore the drug-related death statistics only gives limited information in post-mortem findings of a particular drug as some cases will be omitted and this will underestimate the impact of drug abuse.

With etizolam well documented in drug related deaths from just examining one year of data, questions still remain over designer benzodiazepines that came immediately after. Pyrazolam, diclazepam and flubromazepam emerged closely after etizolam, see Figure 1. It is not clear from the yearly report on drug-related deaths what trends these drugs followed and if they are present in the Scottish drug users dying from drug related deaths or any death for that matter. The lack of data in this area leads to uncertain toxicological interpretation, as there is a lack case data to consult. From the high number of etizolam positives in post-mortem samples, inferences can be drawn that one or more of the drugs which followed on from etizolam would be popular amongst drug users in Scotland and therefore present in post-mortem blood samples especially as these drugs were not controlled by any legislation initially and were sold in shops and online.

1.9.2. Designer benzodiazepine prevalence

In general, drug prevalence is very difficult to quantify and ascertain. Self-reporting studies and defined sub-populations such as individuals requiring treatment from emergency departments can be a way to estimate this.

The National Poison Data System (NPDS) in the USA collated the data from reported intentional exposures of single-agent designer benzodiazepines. The NPDS had been tracking etizolam exposures from 2007 but not the other designer benzodiazepines; these were added in 2016. The study results show that from 2014 to 2017 there have been 162 instances of etizolam exposure, 4 instances of diclazepam and 3 of flubromazepam (Carpenter *et al.*, 2019)

An article published in 2018 detected 15 designer benzodiazepines, from 2012 to 2016, in individuals presenting as intoxicated to Swedish emergency departments. The study found that there was an increase in positive designer benzodiazepines from 4% in 2012 to 19% to 2015. Amongst other designer benzodiazepines, the study noted that flubromazolam had been detected in urine samples in 92 instances, pyrazolam in 33 instances, flubromazepam was also detected in 33 instances, etizolam in 20 instances and phenazepam in 1 instance. (Bäckberg *et al.*, 2019) There is no data to suggest the designer benzodiazepines were all individual cases but it was noted that 89% of cases tested had other drugs present. No metabolites appeared to be tested for, with the exception of 3-hydroxyphenazepam for phenazepam. The study concluded that the absence of a parent compound counted as a negative result, so the presence of 3-hydroxyphenazepam was not a positive for phenazepam. The article did not consider 3-hydroxyphenazepam is also a drug, not just a metabolite and its presence may indicate 3-hydroxyphenazepam use not phenazepam use. There are likely positive instances that have been missed in this sample population due to a lack of metabolites tested. No concentration data was included in the article.

Another article described an unresponsive 30-year old male who was admitted to an emergency department in Florida. (Runnstrom *et al.*, 2019) The urine screen (not further described) was positive for lorazepam and cannabis. After ten days in hospital the patient had recovered and reported he had ingested 240 mg of diclazepam in liquid form. There were no concentrations in tissue samples reported for this case, presumably as the emergency department were not aware

of what the patient had taken however the lorazepam, a metabolite of diclazepam in urine indicates diclazepam exposure. (Runnstrom *et al.*, 2019)

A study in China carried out retrospective analysis on the records of forensic cases from 2017. The study found six positive benzodiazepine cases in the biological samples of drug-facilitated sexual assault (DFSA) victims. There were also six diclazepam-positive liquor samples from DFSA cases. (Xiang *et al.*, 2018) However it was not clear if the liquor was from the same cases as the biological samples. Another Chinese study describes detecting diclazepam in a milky fluid from a DFSA case. (Xiang *et al.*, 2018)

Table 6 displays the studies that have reported a designer benzodiazepine concentration in a human tissue sample. The median concentration is shown when the study had multiple positive results. The concentration without a median is from one positive case or self-administration studies.

A recent Norwegian study collated the blood concentrations from designer benzodiazepines from both living offenders such as apprehended drivers and post-mortem samples. (Heide *et al.*, 2020) These cases were from June 2016 and September 2019. The median concentrations from the drugs in this study are shown in Table 6. An interesting aspect of this study is that in 25 of the living offender cases there was no other drugs detected or the concentration of these drugs were not considered to be the cause of impairment, this makes an assessment of impairment possible and can be related to the concentration of the drug found in the blood. The clinical impairment conclusion ranged from not impaired to considerably impaired with mildly and moderately impaired in between. Diclazepam was the most commonly detected drug in 16 out of the 25 cases discussed. The concentrations for the not impaired individuals were 0.0054, 0.0077, 0.0089 and 0.032 mg/L. The concentrations for the mildly impaired individuals were 0.0051, 0.0077 and 0.035 mg/L. The concentrations for the moderately impaired individuals were 0.011, 0.016, 0.019, 0.032, 0.045, 0.061mg/L. The concentrations for the considerably impaired individuals were 0.014, 0.035 and 0.048 mg/L. The median concentration of not impaired was determined to be 0.0083 mg/L and 0.025 mg/L for impaired.(Heide *et al.*, 2020) The study acknowledges different physicians carried out the clinical impairment assessment and therefore the consistency in this assessment is unknown. The authors were not privy to information regarding time of consumption, dosing or

past drug use history. Diclazepam was detected in 13 out of the 21 post-mortem cases in this study, the concentrations ranged from 0.0018 to 0.032 with a median of 0.0032 mg/L. The other designer benzodiazepines detected in post-mortem blood were phenazepam (n=5), etizolam (n=2) and flubromazolam (n=1). The low number of etizolam positives in post-mortem blood, 2 out of 6,500 cases, is very interesting as it is so low compared to the findings in Scotland where etizolam was the most implicated designer benzodiazepine in drug-related deaths, see section 1.9.1. The authors in the study did not know the cause of death so it was not possible to consider the drugs role in this way.

A 2019 American study found after re-analysing 33 PM samples for designer benzodiazepines, they identified analytes previously missed in 5 samples, including diclazepam, delorazepam, flubromazolam and clonazolam. (Mei *et al.*, 2019) This demonstrates how novel drugs are often missed in routine analysis.

Table 6: Reported designer benzodiazepine concentrations in human samples

Author(s)	Year	Designer benzodiazepine	Matrix	Concentration (mg/L)	AM/PM*	Number of cases
Moosmann *et al* (Moosmann, Bisel and Auwärter, 2014)	2014	Diclazepam	Serum	0.003	AM	1
Høiseth *et al* (Høiseth, Tuv and Karinen, 2016)	2016	Diclazepam	Blood	0.013 -median	AM	15
Lehmann *et al* (Lehmann *et al.*, 2019)	2019	Diclazepam	Blood	0.001	PM	1
Lehmann *et al* (Lehmann *et al.*, 2019)	2019	Diclazepam	Urine	0.001	PM	1
Heide *et al* (Heide *et al.*, 2020)	2020	Diclazepam	Blood	0.0094-median	AM	334
Heide *et al* (Heide *et al.*, 2020)	2020	Diclazepam	Blood	0.0032-median	PM	13
Mei *et al* (Mei *et al.*, 2019)	2019	Delorazepam	Blood	0.037-median	PM	4
Høiseth *et al* (Høiseth, Tuv and Karinen, 2016)	2016	Etizolam	Blood	0.0053-median	AM	14
Heide *et al* (Heide *et al.*, 2020)	2020	Etizolam	Blood	0.054-median	AM	40
Heide *et al* (Heide *et al.*, 2020)	2020	Etizolam	Blood	0.026-median	PM	2
Moosmann et al (Moosmann *et al.*, 2013a)	2013	Flubromazepam	Serum	0.078	AM	1
Høiseth *et al* (Høiseth, Tuv and Karinen, 2016)	2016	Flubromazepam	Blood	0.055-median	AM	24
Koch *et al* (Koch *et al.*, 2018)	2018	Flubromazepam	Blood	0.83	AM	1
Heide *et al* (Heide *et al.*, 2020)	2020	Flubromazepam	Blood	0.037-median	AM	5
Høiseth *et al* (Høiseth, Tuv and Karinen, 2016)	2016	Flubromazolam	Blood	0.012 -median	AM	25
Łukasik-Głębocka *et al* (Łukasik-Głębocka *et al.*, 2016)	2018	Flubromazolam	Serum	0.059	AM	1
Heide *et al* (Heide *et al.*, 2020)	2020	Flubromazolam	Blood	0.0056-median	AM	20
Heide *et al* (Heide *et al.*, 2020)	2020	Flubromazolam	Blood	0.052	PM	1
Shearer *et al* (Shearer *et al.*, 2015)	2015	Phenazepam	Blood	0.10 –median in DRDs 0.09 median in non DRDs	PM	54 89
Heide *et al* (Heide *et al.*, 2020)	2020	Phenazepam	Blood	0.022-median	AM	138
Heide *et al* (Heide *et al.*, 2020)	2020	Phenazepam	Blood	0.0059-median	PM	5
Crichton *et al* (Crichton *et al.*, 2015)	2015	Phenazepam	Blood	0.097 - median	PM	29
Moosmann *et al* (Moosmann *et al.*, 2013b)	2013	Pyrazolam	Serum	0.051	AM	1
Høiseth *et al* (Høiseth, Tuv and Karinen, 2016)	2016	Pyrazolam	Blood	0.074	AM	1
Lehmann *et al* (Lehmann *et al.*, 2019)	2019	Pyrazolam	Blood	0.028	PM	1

*AM = Ante-mortem, PM =Post-mortem

With the exception of Shearer *et al*, Crichton *et al* and Heide *et al* there is a lack of information in the scientific literature of the post-mortem blood concentrations of designer benzodiazepines in multiple cases. Diclazepam and pyrazolam post-mortem blood concentrations are discussed by Lehmann *et al* but in a single case and Mei *et al* discusses PM findings but again only in five case therefore the primary goal of this study is to provide typical blood concentrations found at post-mortem in order to aid toxicologists and pathologists in the interpretation. Firstly, it must be determined if an ELISA screening method is able to detect these drugs in

order to flag the positive samples for confirmation. The secondary goal is to ascertain the use of benzodiazepines in sub-populations who are at high risk of using these drugs but are under conditions where they should not use them. These are living participants who have given a urine sample, which is non-invasive unlike blood.

1.10. Analytical testing of benzodiazepines

As benzodiazepines are commonly prescribed throughout the world a large number of analytical methods in human tissue have been described in published literature. Table 7 shows an overview of the methods for the designer benzodiazepines, including the instrumentation and extraction method used.

Systematic toxicological analysis follows a methodical approach of detection, identification and quantification. (Negrusz and Cooper, 2013) It is important to understand if the methods currently used in toxicology laboratories are able to detect newly emerged benzodiazepines. Laboratories commonly carry out the first step of detection by a screening method such immunoassay before any confirmatory tests are commenced. The number of confirmatory tests can be reduced in this way, as the negative samples will not require further analysis. As immunoassay techniques utilise antibodies targeting the general structure of a drug, there is a lack of selectivity, and therefore they typically can detect a drug group and not a specific compound. This concept is promising as structural analogs similar to the target drug will not give a negative response and therefore new designer drugs may give a positive immunoassay result. This is further discussed in chapter 2.

After presumptive detection, the specific analyte(s) must be confirmed and quantified typically by either liquid chromatography tandem mass spectrometry (LC-MS/MS) or Gas Chromatography Mass Spectrometry (GC-MS) but before this, a suitable sample preparation method must be determined. Sample preparation techniques depend on factors such as sample matrix, the aim of the analysis and the type of instrumentation used. Two common sample preparation techniques for extracting benzodiazepines are Solid Phase Extraction (SPE) and Liquid Liquid extraction (LLE). SPE works by exploiting the differences in polarity between the analyte of interest and the other components in a complex sample. The solid phase is a sorbent within the cartridge, which retains the analyte of

interest. The other interferent components such as proteins can be washed through the sorbent to waste; the target analyte is then eluted from the sorbent and collected to be evaporated and reconstituted into a solvent mixture suitable for injection onto the analytical instrument. The use of SPE is more expensive than using LLE, as the SPE cartridges must be purchased, however SPE is more selective, gives cleaner extracts, increased extraction recovery and avoids emulsion formation. LLE works by using two different immiscible liquids, a polar solution usually an aqueous buffer containing sample is mixed with a non-polar solvent in which the analyte is soluble in. The pH of the buffer is controlling the ionisation of the analyte of interest and this helps to facilitate the transition from one liquid to the other. After mixing and centrifugation the solvent portion now containing the analyte of interest can be removed for injection or evaporation then reconstitution in a solvent mixture suitable for injection onto the analytical instrument. The aqueous portion containing the interferant components is discarded. LLE is a broad extraction technique with much less selectivity than SPE. This can be an advantage in the detection of new structurally related compounds as they are likely to also be extracted, whereas they may not elute from a specific SPE cartridge. LLE as an extraction method has been chosen by most for the designer benzodiazepines likely because it is a broad extraction, see Table 7.

Urine analysis of benzodiazepines may require enzymatic hydrolysis steps before the extraction takes place if the conjugated metabolites are not included in the analytical method. As shown the section 1.7, the compounds will undergo glucuronidation in order to excrete them from the body. Enzymatic hydrolysis cleaves the glucuronide freeing the analyte and therefore making it more detectable. Including a hydrolysis step adds to the assay turnaround time, as an incubation period is required. There is also the issue of different metabolites having a variable hydrolysis rate; incomplete hydrolysis will lead to a false decreased concentration or a potential false positive for an analyte. (Johnson-Davis, 2018) Beta-glucuronidase from *Helix Pomatia* is commonly used in benzodiazepine urinalysis as it gives a high yield for a number of benzodiazepines (Fu *et al.*, 2010) which is crucial as a urine sample often contains multiple analytes.

Table 7: Overview of designer benzodiazepine analysis methods published

Author(s)	Analyte(s)	Matrix	Sample Preparation	Instrumentation	Internal standard	LOD (mg/L)
Crichton *et al* (Crichton *et al.*, 2015)	3-hydroxyphenazepam Phenazepam	Blood Plasma Urine Vitreous Muscle Liver Brain	LLE	LC-MS/MS	Diazepam-D5	0.0003 0.007 (in plasma)
Meyer *et al* (Meyer *et al.*, 2016)	Clonazolam Meclonazolam Nifoxipam	Urine	LLE	LC-MS	Methamphetamine-D5 Pethidine-D4	n/a
Høiseth *et al* (Høiseth, Tuv and Karinen, 2016)	Clonazolam Diclazepam Etizolam Flubromazepam Flubromazolam Pyrazolam	Blood	LLE	UPLC-MS/MS	Diazepam-D5	0.0014 0.0016 0.0014 0.0033 0.00037 0.0035
Heide *et al* (Heide *et al.*, 2020)	Clonazolam Diclazepam Etizolam Flubromazepam Flubromazolam Phenazepam	Blood	LLE	UHPLC-MS/MS	Diazepam-D5 Nitrazepam-D5 Flunitrazepam-D7 Diclazepam-D4 Etizolam-D3	0.0014 0.0016 0.0014 0.0033 0.00037 0.0017
Mei *et al* (Mei *et al.*, 2019)	Clonazolam Diclazepam Delorazepam Flubromazepam Flubromazolam Meclonazepam Phenazepam Nifoxipam Pyrazolam 3-hydroxyphenazepam	Blood	SPE	LC-MS/MS	Diazepam-D5 Chlordiazepoxide-D5 DMD-D5 Temazepam-D5	0.0005 0.0005 0.0005 0.0005 0.0005 0.0005 0.0005 0.0005 0.0005 0.0005
Moosmann *et al* (Moosmann, Bisel and Auwärter, 2014)	Diclazepam	Plasma Urine	Hyrolysis by beta-glucuronide LLE	LC-MS/MS	Diazepam-D5 Lorazepam-D4 DMD-D5 Temazepam-D5	0.00025
Bergstrand *et al* (Bergstrand, Helander and Beck, 2016)	Diclazepam Pyrazolam Flubromazepam Meclonazepam Phenazepam Etizolam Nifioxipam Deschloroetizolam Clonazolam Flubromazolam	Urine	Hyrolysis by beta-glucuronide Direct injection	LC-MS/MS	Estrazolam-D5 Temazepam-D5	0.0020 0.0040 0.0025 0.0010 0.0050 0.0020 0.0100 0.0020 0.0050 0.0020
Fracasso *et al* (Fracasso *et al.*, 1991)	Etizolam	Plamsa Urine	LLE	HPLC	Alprazolam	0.001
Nakamae *et al* (Nakamae *et al.*, 2008)	Etizolam	Plamsa Urine	SPE	GC-MS/MS	Fludiazepam	n/a
Moosmann *et al* (Moosmann *et al.*, 2013a)	Flubromazepam	Plasma Urine	LLE	LC-MS/MS	DMD	0.001
Kintz *et al* (Kintz *et al.*, 2017)	Metizolam	Urine	LLE	LC-MS/MS	Methyl-clonazepam OH-ethylthoephyllin	0.00025
Stephenson *et al* (Stephenson, Golz and Brasher, 2013)	Phenazepam	Plasma	LLE	LC-MS/MS	Diazepam	0.012
Moosmann *et al* (Moosmann *et al.*, 2013b)	Pyrazolam	Plasma Urine	LLE	LC-MS/MS	Alprazolam	0.001

The use of GC-MS requires a derivatisation step post-extraction. Derivatisation is the modification of a compound to make it more suitable for analysis. Derivatisation is essential to form a stable benzodiazepine complex, which can be analysed by GC-MS without decomposing on the GC column due to the high temperature required for elution. The lack of a suitable universal derivatisation

agent for benzodiazepines is an issue and using a LC technique avoids any derivatisation problems. (Segura *et al.*, 2001)

LC-MS/MS is now often the preferred analytical instrument for forensic toxicology laboratories. LC-MS/MS is able to analyse larger, polar compounds than GC-MS(/MS) as there is no need to impart high temperatures, the sample preparation can also be faster and cheaper, for example there is no need to chemically modify compounds as is required with the derivatisation. However the initial cost of purchasing an LC-MS(/MS) instrument is much more costly than a GC-MS(/MS). (Drummer and Gerostamoulos, 2014) The weekly maintenance of a GC-MS is very quick and easy as it usual just requires a change of liner and septum with frequent auto-sampler wash bottle changes, in contrast a heavily used LC-MS needs mobile phases prepared every few days and a weekly clean as there is a fast drop in sensitivity when regions around the source entry become dirty. In addition, ion suppression or enhancement is an issue specific to the technique (Furey and Moriarty, 2011) (see section 3.4.6 in chapter 3), if either of these occur then changes in the protocol such as sample dilution, reagent changes, using different ionisation (APCI rather than ESI) and chromatographic changes may have to be investigated. The use of multiple reaction monitoring in LC-MS(/MS) has led to the ability to target large numbers of drugs in a sample simultaneous in a relative short period of time however problems have been encountered where a metabolite of a drug has the same mass/charge ratio as one of the target analytes within the analytical method, particularly if they also have the same retention time. This was observed when a single MRM transition was used for the identification of tramadol leading to false positives in patients who had been treated with venlafaxine. This false positive has due to metabolite, *O*-desmethylvenlafaxine and tramadol being isobaric and as the method had a short run time (6.5 minutes) they were co-eluting. (Allen, 2006) This issue is solved with using more than one transition, using a complementary technique in addition to LC-MS(/MS) (i.e a UV spectrum will tell them apart) (Furey and Moriarty, 2011) and baseline resolution can be attempted once an issue like this one is known. (Allen, 2006) LC-MS does not have established spectral libraries in the way that GC-MS does which is a huge advantage to using GC-MS. (Drummer and Gerostamoulos, 2014) Overall, both instruments are extremely valuable to the field of forensic toxicology and access to both is preferable and complementary.

It is accepted that LC-MS(/MS) is more appropriate than GC-MS(/MS) to analyse benzodiazepines due to their non-volatile and polar nature (Nakamura, 2011; Drummer and Gerostamoulos, 2014) and the requirement to determine low concentrations of benzodiazepines in cases such as a drug-facilitated sexual assault (DFSA) has also seen methods move from GC-MS to LC-MS(/MS). (Persona *et al.*, 2015)

This work used LC-MS/MS to analyse benzodiazepines in urine and post-mortem blood samples.

The LC-MS/MS instrument is a High Pressure Liquid Chromatography (HPLC) system combined with a mass spectrometer (MS). There are two main components which make up the mass spectrometer; the ion source, which causes the ionisation of the molecules and the mass analyser, which is responsible for filtering the ions by their mass to charge ratios (*m/z*). (Agilent Technologies, 2001)

The discovery of Atmospheric Pressure Ionisation (API) improved the interface between the HPLC and mass spectrometer leading to ions being obtained in a more reliable manner. There are two main types of API.

1.10.1. Electrospray ionisation

In electrospray ionisation (ESI) gas phase ions are converted from solution phase analytes. Following elution from the LC system, the mobile phase carries the analytes into the ESI to the capillary tip where a high voltage is applied. Nebuliser gas, often nitrogen, flows outside the capillary and sprays the sample; this turns the sample and mobile phase into a fine aerosol. The droplets within the aerosol are charged with the same polarity as the voltage applied. The solvent continues to evaporate under temperature control and as these charged particles move around, causing an increasing electric field on the droplet surface until fission occurs and the droplets become smaller more stable droplets. This cycle of fission is repeated until they are small enough that the sample ions are liberated into the gas phase. (Agilent Technologies, 2001; McMaster, 2005) Figure 15 shows a schematic diagram of an ESI source.

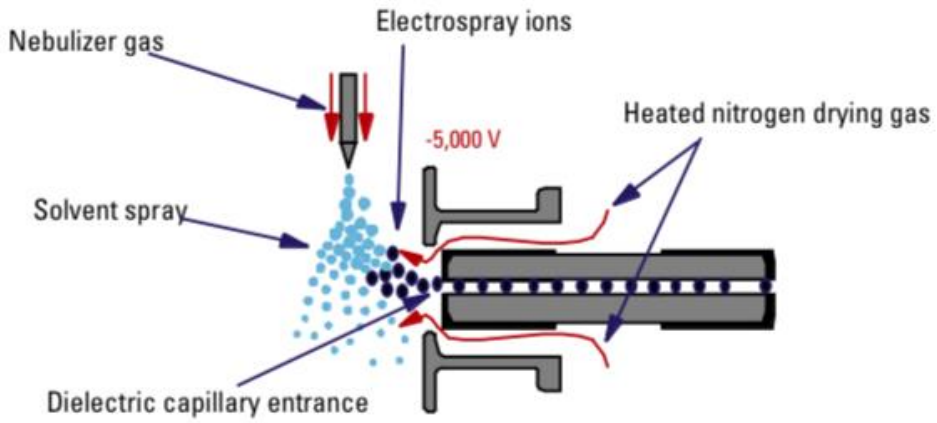

Figure 15: ESI diagram
(reproduced with permission from Agilent)

1.10.2. (Atmospheric Pressure) Chemical ionisation

In (Atmospheric Pressure) chemical ionisation (APCI), the sample and solvent are vaporised by spraying the LC eluent under temperature, which can be heated up to 400°C, using an inert gas, usually nitrogen. Stable reaction ions are then generated by discharge from the corona ionising the solvent ions. Sample molecules are then ionised by the transfer of protons between the reaction ions and the sample molecules.(Agilent Technologies, 2001) Figure 16 shows a schematic diagram of an APCI source.

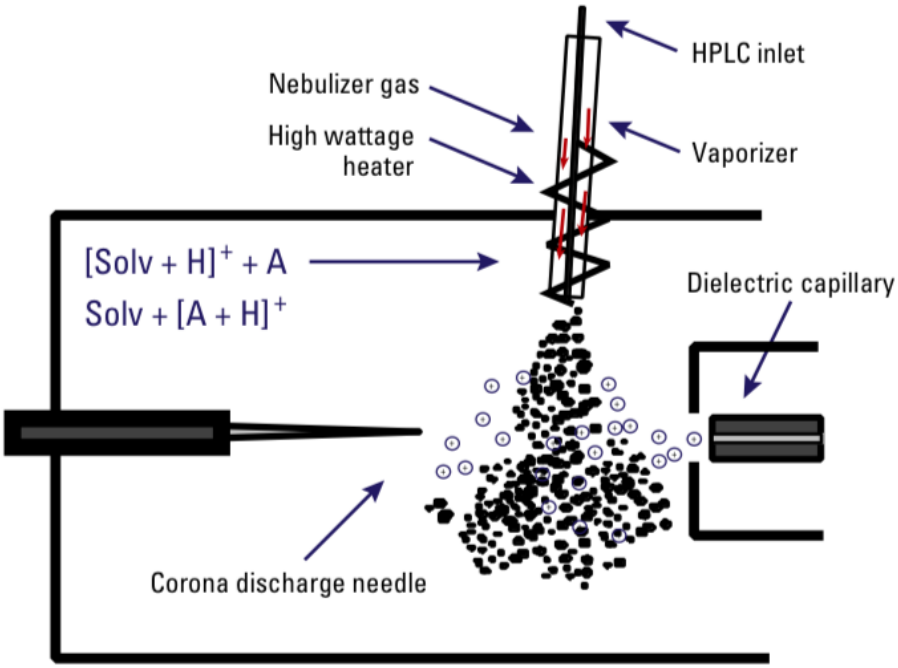

Figure 16: API-CI diagram
(reproduced with permission from Agilent)

1.10.3. Mass analysers

Two common mass analysers used are a triple quadrupole and ion trap. The quadrupole analyser uses four parallel rods arranged in a square formation, the sample ions travel down the tunnel created by the rods. Voltages applied to the rods create the electromagnetic fields and changing radio frequency signals sweep the ions along with each frequency selecting a different mass to charge ratio. This gives the ions a stable path to the detector, the ions strike the detector surface generating a signal, which is amplified and sent to the computer to compose the Total Ion Count (TIC). This kind of analyser can be operated in a variety of modes; there are four main scans for multiple applications of the LC-MS/MS system. The product ion scan uses a selected precursor ion in quadrapole 1 (Q1) and focuses it into the collision cell (Q2) where a process known as collision induced dissociation occurs, this is when inert gas such as nitrogen collides with ions causing fragmentation, the fragments are then filtered by their *m/z* in quadrapole 3 (Q3) producing a spectrum of product ions. The precursor ion scan is used to identify an ion with a chosen *m/z* in Q3, Q1 scans over the desired *m\z* range then into Q2 for fragmentation then Q3 is held static on the desired *m/z* resulting in the precursor ion spectrum. The third scan type is constant neutral

scanning. This is utilised to monitor particular compounds by setting a mass offset between Q1 and Q3, which will both scan across the whole *m/z* range. Q2 will allow only the ions that differ by the pre-set mass units into Q3, this is the neutral fragment, for example alcohols could be monitored in this way by setting a loss of 18 Da (water) between Q1 and Q3. The forth type of scan mode is multiple reaction monitoring (MRM). In MRM mode, the MS/MS is set to detect the precursor and product ions of a known compound. The targeted approach of MRM makes it a highly sensitive and specific application.

A ion trap systems works on a similar principle to the quadrupole but instead of filtering streaming ions, the ion trap holds them in a three dimensional space. The ions sit between two electrodes and are ejected to the detector or are stored there based on the radio frequency applied. (McMaster, 2005; Agilent Technologies, 2001)

1.11. Aims and objectives

The primary aim of this project was to determine the typical concentrations of diclazepam (and two of its metabolites, delorazepam and lormetazepam), flubromazepam and pyrazolam in post-mortem blood in order to aid toxicological interpretation. In order to assess the use of these drugs in this cohort the following objectives were set.

1. Ascertain if designer benzodiazepines in the post-mortem confirmation methods will trigger a positive response in an ELISA screening method.
2. Validate the analytical quantitative SPE - LC-MS/MS method to detect diclazepam (and two of its metabolites, delorazepam and lormetazepam), flubromazepam and pyrazolam in post-mortem blood.
3. To apply the validated method to post-mortem blood samples to determine typical concentrations in this sample set, collect demographic data relating to these cases to determine the prevalent populations and how their presence is implicated in the cause of death.

The secondary aim of this study was to determine the use of benzodiazepines in urine samples from different Scottish high-risk sub-populations. In order to achieve this the following objectives were set.

1. To develop and partial validate a benzodiazepine LLE - LC-MS/MS qualitative method for urine samples.
2. To apply this analytical method to prison samples, drug court samples and psychiatric urine samples in order to assess if these drugs are used.

2. ELISA cross-reactivity determination of phenazepam, etizolam, diclazepam, delorazepam, pyrazolam and flubromazepam

2.1. Introduction

2.1.1. Immunoassay

An immunoassay is a biochemical test that utilises an antibody to detect the presence of a compound (an antigen). Antibodies' role in nature is to detect disease, and this has been manipulated in order to use their discriminatory powers to detect other molecules such as drugs, hormones or marker proteins. Since the 1970s, pregnancy tests have used immunoassay as a fast and convenient method to detect the human chorionic gonadotropin (hCG) hormone in urine. (Horwitz and Lee, 1978)

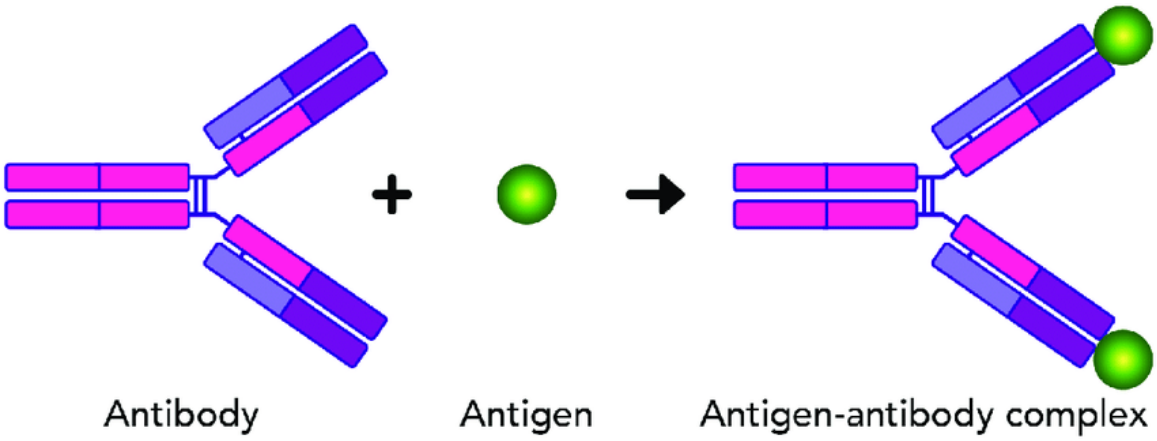

Figure 17: The antibody – antigen reaction
(reproduced with permission from Jiménez-Martínez - Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/Antigen-antibody-reaction-Each-antibody-is-able-to-bind-its-specific-antigen-forming_fig1_320265684)

When an antibody and its specific antigen meet, an *immune complex* forms, see Figure 17. Normally this process is undetectable therefore a *label* is required. A *label* is a sensor that has some kind of activity that produces an output and can then be read in a laboratory (e.g. radiation, fluorescence or an enzyme reaction with a *substrate* producing a colour change).

2.1.2. Immunoassays in forensic toxicology

Prior to immunoassay, drug detection in biological samples was carried out using techniques such as microcrystalline testing and Thin Layer Chromatography (TLC). Microcrystalline tests work by introducing the sample to a specific reagent,

which will form unique crystals if the suspected drug is present; these are then compared to the crystals formed by reference material under a microscope. (Elie *et al.*, 2009) TLC is a simple chromatography technique in which compounds will separate out onto a stationary phase depending on their affinity for the mobile phase. Immunoassay is useful in toxicology as it indicates which drug group is present, but it is not a complete tool as it cannot detect the specific drug, therefore further investigation is required. Immunoassay can sometimes give false positives, when another substance gives a similar response to the target substance, therefore all positive results must be confirmed by a second test. There can also be instances of false negative results therefore it is imperative to manufacture a device which has the required sensitivity, whilst not returning too many false positive results. The introduction of immunoassays to forensic toxicology had a direct impact on the speed at which samples could be screened. (Chung and Choe, 2017; Moody, 2006)

Due to the possibility of false-positive results, immunoassays are considered presumptive only tests; the results cannot be used in legal proceedings and must be confirmed by a more sophisticated technique. Immunoassay use is appropriate in situations such as workplace drug testing where a high percentage of samples are expected to give negative results. There have been examples of the use of immunoassay with no confirmation testing in legal proceedings in recent years. The Motherisk Drug Testing Laboratory in Canada tested hair samples from parents for Child Protection Services in Ontario. All hair samples were tested using ELISA from January 2005 to August 2010 by the laboratory; the tests most frequently carried out were cocaine, benzoylecgonine and delta-9-tetrahydrocannabinol (THC). In this time period ELISA results were reported without any further confirmation tests. The laboratory was responsible for testing around 2,000 hair samples per year. This finding along with the lack of standard operating procedures, documentation and general lab management led to an independent review on the behalf of the Attorney General which found the laboratory "inadequate and unreliable for use in child protection and criminal proceedings and that the Laboratory did not meet internationally recognised forensic standards." (Lang, 2015)

A recent article described how a newly developed immunoassay, based on a biochip rather than the lateral flow technique, could be used as part of the post-

mortem procedure in order to make decisions on whether confirmation tests are required. The device used in this study showed a high degree of agreement with confirmatory techniques, >98% agreement when used on femoral blood. (McLaughlin *et al.*, 2019) There has been a move away from immunoassay in forensic toxicology in recent years aided by the evolution of other screening techniques using advanced instrumentation such as Time of Flight (TOF). The high cost of consumables also makes immunoassay a less attractive option.

Calibrators are used to produce a calibration curve, which can be compared to the unknown samples. These concentrations are not reported, but can be used to determine if dilutions are required for positive samples. The lowest calibrator or control is used as the *cut-off* to give a decision point between negative and presumptively positive samples. (Moody, 2006) In line with other quality procedures in forensic toxicology, a drug-free sample in the same matrix (a *blank*) should be included in every immunoassay screen. At least one positive control should also be analysed to demonstrate that the assay is working correctly. Biological samples that screen presumptively positive are typically confirmed by a hyphenated mass spectrometric technique (e.g. LC-MS/MS or GC/MS), which will identify the specific drug and/or metabolite present. When isobaric compounds are a concern, high performance liquid chromatography with a diode array detector (HPLC-DAD) may be employed, as the UV spectra will distinguish between them. (Furey and Moriarty, 2011)

Samples that are negative do not usually require any further testing for that drug group. This can be potentially risky strategy for the groups of drugs that are expanding due to the rise in NPS such as fentanyls and benzodiazepines. Due to the way drug antibodies are raised, they may partially bind to drugs that are chemically similar to their specific *target*. This is known as *cross-reactivity*. An article by Guerrieri *et al*, explored the cross-reactivity of the fentanyl analogs with an existing fentanyl targeted kit, they were found to give good cross reactivity with the exception of carfentanil due to an additional carbomethoxy group present in its structure. (Guerrieri *et al.*, 2019) Therefore when reporting results care must be taken to understand what drugs the kit is able to screen for. The ELISA kit used in this study is specific to oxazepam but is used for the detection of other benzodiazepines. Oxazepam is a metabolite of diazepam, desmethyldiazepam, temazepam, chlordiazepoxide and a range of other lesser-encountered

benzodiazepines as well as a parent drug itself. The benzene ring and its positional groups are the area of the molecule responsible for binding. It is important to know the level of cross-reactivity of new drugs to existing immunoassay kits due to the possibility that not all designer benzodiazepines will bind. This could result in the laboratory failing to identify the drug if immunoassay alone is relied upon. A study carried out in 2017 assessed the cross-reactivity of 13 designer benzodiazepines (clonazolam, deschloroetizolam, diclazepam, estazolam, etizolam, flubromazepam, flubromazolam, flutazolam, 3-hydroxyphenazepam, meclonazepam, nifoxipam, phenazepam, and pyrazolam) in urine in four different immunoassay kits. The authors evaluated the CEDIA, EMIT II Plus, HEIA, and KIMS II immunoassays. They found that all 13 drugs tested in spiked urine had high cross-reactivity to the benzodiazepines antibody and this was confirmed when authentic positive urine samples were tested. They found that flutazolam gave the lowest cross-reactivity, (3, 4, 13 and 41% over the four kits tested, most other analytes tested were over 50% for at least three out of the four kits), which may be explained by its structure; as it is the most divergent from the other benzodiazepines, see Figure 18. They found the CEDIA and KIMS II immunoassays to be the most cross-reactive, and EMIT II Plus to be the least cross-reactive. (Pettersson Bergstrand *et al.*, 2017)

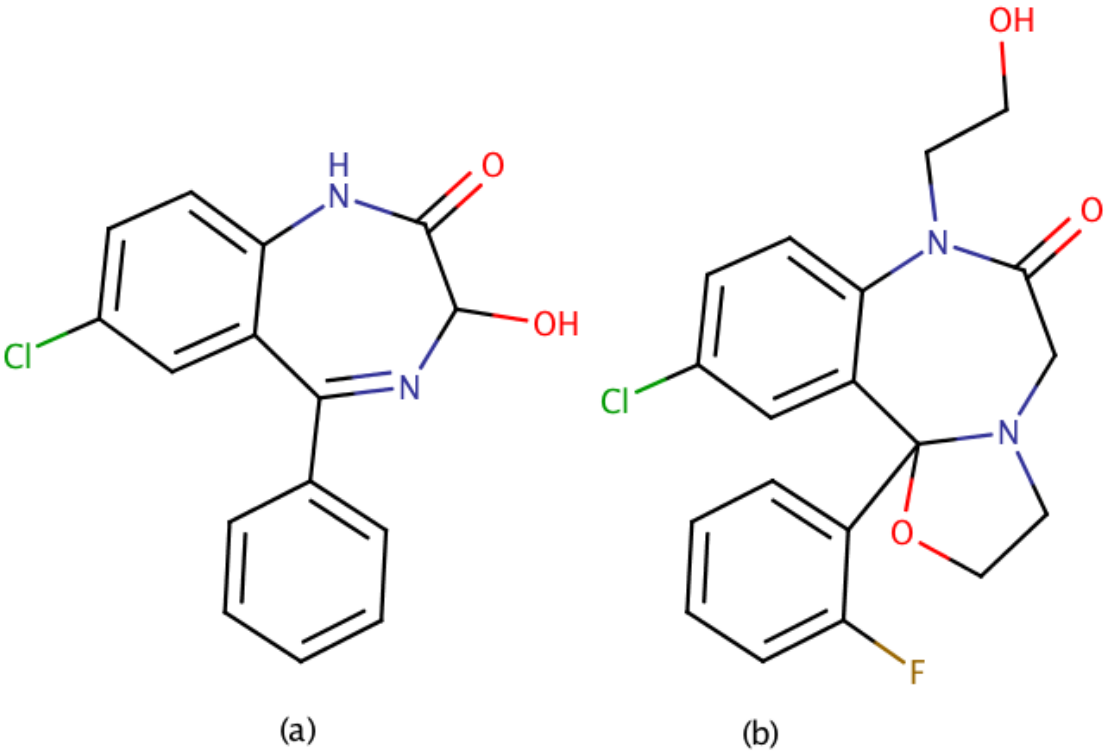

Figure 18: Oxazepam (a) and flutazolam (b) structure

2.2. Aim

The aim of this study was to determine if phenazepam, etizolam, diclazepam, delorazepam, pyrazolam and flubromazepam would cross react with the Immunalysis® Benzodiazepine ELISA kit. These drugs were chosen, as there was increasing evidence from various agencies such as the police and online searches that these drugs may be abused in Scotland. It is crucial for a toxicology laboratory to be aware if their screening techniques are adequate and do not fail to identify positive samples which need further confirmatory testing.

2.3. Materials and Methods

2.3.1. Materials

Phenazepam, etizolam and oxazepam were purchased from Sigma Aldrich, UK. Pyrazolam and diclazepam were purchased from Chiron AS, Trondheim, Norway. Flubromazepam and delorazepam were purchased from LGC Standards, Teddington, UK. Blank blood used was purchased from the Scottish National Blood Transfusion Service, Gartnavel Hospital, Glasgow, UK. The Immunalysis® Benzodiazepine ELISA kit and the pH 7.0 phosphate buffered saline (PBS) were purchased from Alere Toxicology, Abingdon, UK (now Abbot). HPLC grade methanol was purchased from VWR, Leicestershire, UK.

Immunalysis® Benzodiazepine ELISA kit contains ready to use reagents. Table 8 explains what is included in the kit and what they do.

Table 8: Immunalysis® Benzodiazepine ELISA kit reagents

Item	Purpose
Plate/well	The plates are composed of 96 wells, which are small round containers coated with the antibody. Each well holds a volume of 350 µL.
Conjugate	The conjugate is an enzyme specific compound which is added to compete with the drug of interest for the antibody binding site.
TMB substrate	3,3',5,5'-tetramethylbenzidine and peroxide in a buffer. The TMB substrate attaches to the conjugate and provides the label, this gives the colour change reaction.
Stop solution	1 N hydrochloric acid, the stop solution, halts and stabilises the reaction caused by the TMB substrate. The reaction must be stopped and stabilised to be read by the spectrophotometer.

2.4. Solution preparation

2.4.1. Stock solutions

Individual stock solutions in methanol containing 10 µg/mL of each individual drug was prepared, see Table 9. Oxazepam was used for the cross-reactivity comparison, as this is the manufacturers target molecule.

Class A volumetric flasks were used to prepare the stock solutions; they were inverted several times to mix then transferred to labelled containers. The stock solutions were stored in the freezer (≤-20 °C).

Table 9: Cross-reactivity calibrator and spiked sample stock solutions preparation

Stock Solutions @ 10 µg/mL	**Drug @ 1 mg/mL** ***100 µl added***	**Drug @ 100 µg/mL** ***1 mL added***	**Solvent** ***Up to 10 mL***
Calibrator	Oxazepam	-	Methanol
Phenazepam	Phenazepam	-	Methanol
Etizolam	Etizolam	-	Methanol
Diclazepam	Diclazepam	-	Methanol
Delorazepam	-	Delorazepam	Methanol
Pyrazolam	Pyrazolam	-	Methanol
Flubromazepam	Flubromazepam	-	Methanol

2.4.2. Working solutions

Individual working solutions (1 µg/mL) were prepared by a 1 in 10 dilution of the stock solutions detailed in Table 9. These were prepared in Class A volumetric flasks, they were inverted several times to mix then transferred to labelled containers.

2.4.3. Calibrator and spiked sample preparation

Blank blood (drug-free) and PBS were both spiked with oxazepam at concentrations of 0, 5, 10, 100 and 300 ng/mL. These were used as calibrators. Blank blood was spiked with phenazepam, etizolam pyrazolam, flubromazepam, diclazepam and delorazepam at the same concentrations (see Table 10 for the preparation details). These concentrations were chosen, as they were in-line with the concentrations used in the FMS in-house method.

Table 10: Cross-reactivity calibrator and spiked sample working solutions preparation

Calibrator/spike sample	Concentration (ng/mL)	Volume of Working Calibrator/Spike Solution (1 µg/mL) (µL)	Volume of PBS /blank blood (µL)
Calibrator 0	0	0	1000
Calibrator 5	5	5	995
Calibrator 10	10	10	990
Calibrator 100	100	100	900
Calibrator 300	300	300	700
Individual drug spike 0	0	0	1000
Individual drug spike 5	5	5	995
Individual drug spike 10	10	10	990
Individual drug spike 100	100	100	900
Individual drug spike 300	300	300	700

A 250 µL aliquot of each calibrator and spiked blood was pipetted into a fresh tube and diluted with 1:4 (1 mL added) with PBS and vortex mixed for five seconds.

2.4.4. Instrumentation

The Miniprep 75 was used to automatically pipette the samples into the wells of the ELISA plate. A Columbus plate washer was used to wash the plates and a Sunrise plate reader was used to read the absorbance of the TMB at a wavelength of 450 nm. The Miniprep 75, plate washer and plate reader are all manufactured by Tecan Group Ltd, Switzerland.

2.4.5. Immunoassay procedure

The calibrators and spiked samples detailed in section 2.4.3 were prepared fresh before the immunoassay procedure, each calibrator and spiked sample was pipetted onto the plate in duplicate. The whole procedure was carried out twice on separate days giving four replicates for each drug.

Figure 19 describes the procedure used for this experiment.

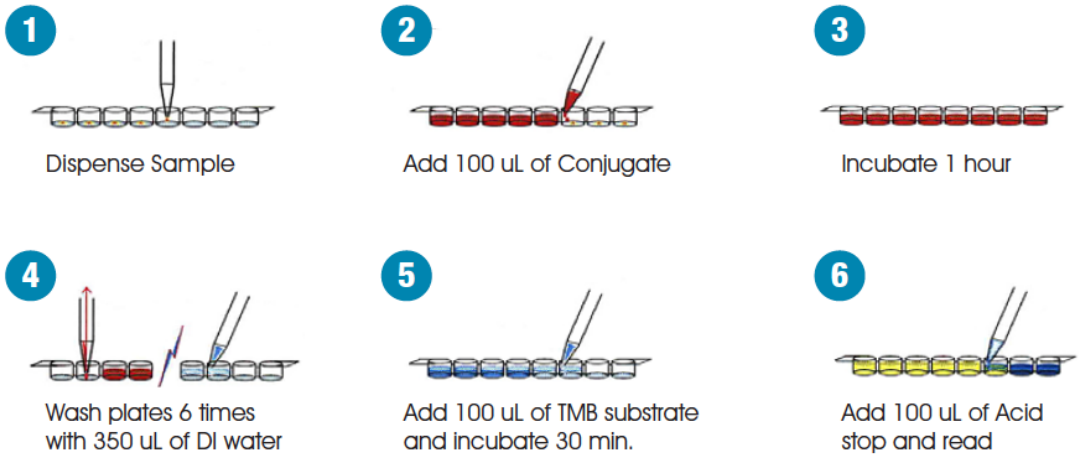

Figure 19: ELISA procedure used
(reproduced with permission from Immunalysis) (www.immunalysis.com., February 2016)

2.4.6. Cross-reactivity determination

The absorbance at 5, 10, 100 and 300 ng/mL for each drug was compared to the absorbance of oxazepam (OXZ) calibrators prepared at the same concentrations, using equation 1.

Equation 1: %Cross-reactivity

$$\%\text{Cross-reactivity} = \left(\frac{\text{mean absorbance of OXZ}}{\text{mean absorbance of drug}}\right) \times 100$$

The higher the %cross-reactivity, the more affinity the drug has for binding to the target. The value can be above 100% as it is a comparison of the drugs binding to the binding of oxazepam; it is possible that some compounds may bind better than oxazepam.

2.4.7. Data handling and statistical analysis

Once results had been generated from the plate reader, they were transferred onto a Microsoft® Excel® (version 14.7.3) spreadsheet and a second toxicologist checked transcription and validity of the results before any calculations were performed. Microsoft® Excel® was then used to calculate the %cross-reactivity, standard deviation and %CV. The Statsplus (AnalystSoft™, version 7.7.31) add on for Microsoft® Excel® was used to conduct a paired two sample t-test to determine if there was a statistical difference between the blood results and PBS results. The spreadsheet was saved on a secure drive to protect the data.

2.5. Results and discussion

The study shows that cross-reactivity of some designer benzodiazepines such as diclazepam and flubromazepam is better in PBS than in blood, see Table 11, matrix effects may explain this. However the blood results show less variation in cross-reactivity across the concentration range for etizolam and pyrazolam. Blood has additional proteins and possibly other drugs present in real sample blood so this presents a more complex challenge for the ELISA kit. However, some blood and PBS results are comparable; etizolam at 10 ng/mL for example shows no issues in the blood matrix. There is no statistical difference between the blood results and the PBS results shown in this study at the 5, 10, and 100 ng/mL concentrations (*p*-values = 0.07, 0.33 and 0.24). The 300 ng/mL concentration gave a higher cross-reactivity for all drugs in PBS compared to blood and this was significant (*p*-values = <0.05). However the cross-reactivity at 300 ng/mL was above 70% for all drugs tested.

Table 11: Cross-reactivity with PBS buffer and blank blood calibrators

Drug	Concentration (ng/mL)	Calibrators in PBS buffer %Cross-reactivity	SD (n = 4)	Calibrators in blood %Cross-reactivity	SD (n = 4)
Phenazepam	5	107	0.01	96	0.01
	10	110	0.02	109	0.03
	100	143	0.01	84	0.01
	300	93	0.02	69	0.02
Mean		***113***		***90***	
SD		***18***		***15***	
%CV		***16***		***17***	
Etizolam	5	111	0.03	100	0.03
	10	96	0.05	96	0.06
	100	84	0.01	126	0.01
	300	143	0.01	107	0.01
Mean		***109***		***107***	
SD		***22***		***12***	
%CV		***20***		***11***	
Pyrazolam	5	90	0.06	94	0.10
	10	75	0.04	88	0.13
	100	139	0.03	82	0.03
	300	111	0.02	81	0.01
Mean		***104***		***86***	
SD		***24***		***5***	
%CV		***23***		***6***	
Flubromazepam	5	98	0.08	91	0.01
	10	103	0.11	98	0.03
	100	82	0.02	75	0.02
	300	105	0.01	73	0.01
Mean		***97***		***84***	
SD		***9***		***11***	
%CV		***9***		***13***	
Diclazepam	5	106	0.08	85	0.04
	10	91	0.07	98	0.03
	100	122	0.04	72	0.04
	300	95	0.00	62	0.01
Mean		***104***		***79***	
SD		***12***		***14***	
%CV		***12***		***17***	
Delorazepam	5	89	0.09	87	0.08
	10	90	0.07	93	0.05
	100	72	0.03	70	0.03
	300	93	0.01	69	0.01
Mean		***86***		***80***	
SD		***8***		***10***	
%CV		***10***		***13***	

The cross-reactivity demonstrated by the results of this study is not unsurprising considering the molecular structure of the target analyte oxazepam compared to the six drugs tested. Figure 20 shows the structure of oxazepam with the six drugs tested in this experiment. Etizolam and pyrazolam have more divergent structures from oxazepam but both still gave high %cross-reactivity, particularly etizolam, which gave very high mean %cross-reactivity results when calibrators were prepared in blood.

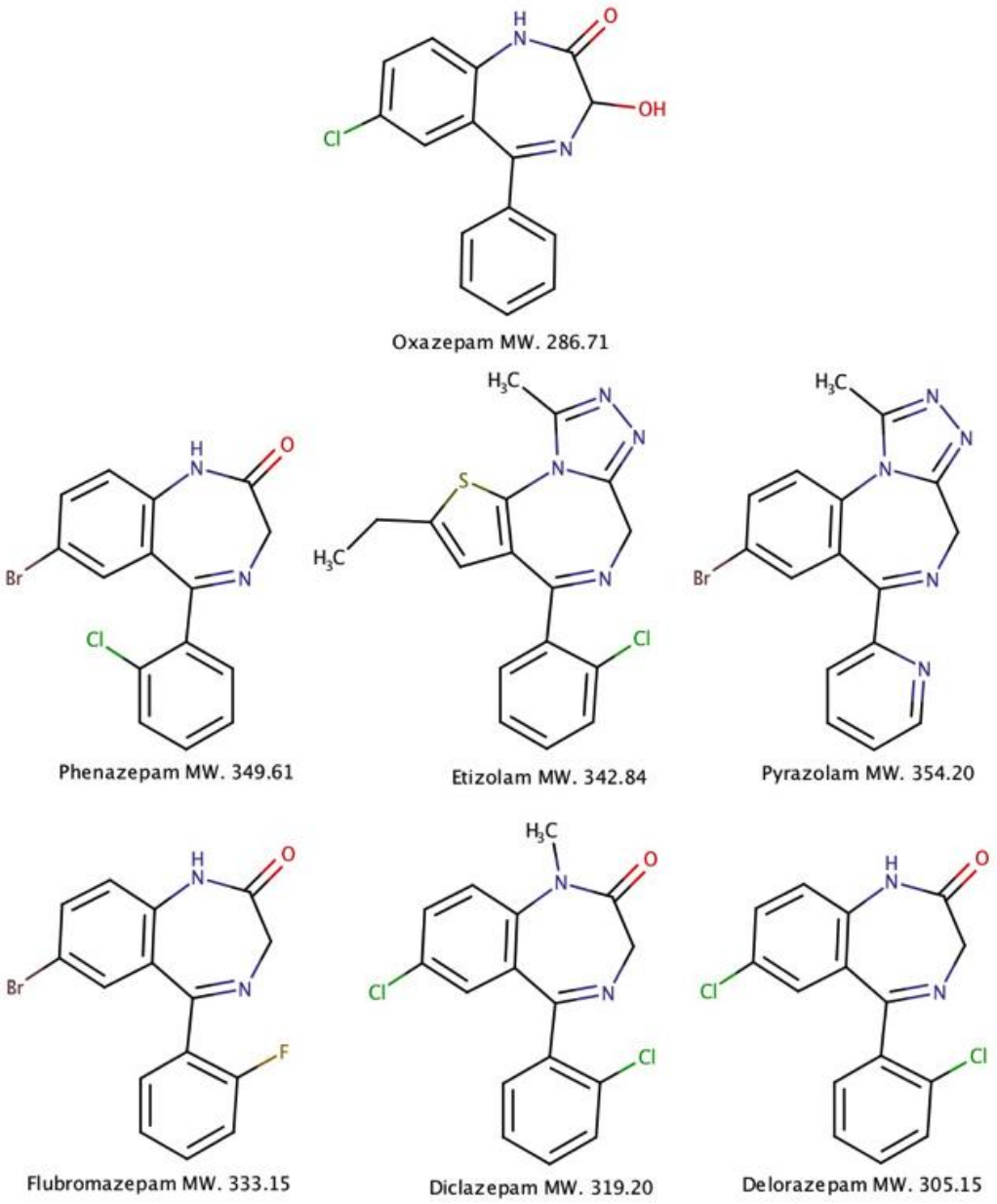

Figure 20: Molecular structure of oxazepam and the drugs tested for cross-reactivity

The cross-reactivity of diclazepam's other metabolites, lorazepam and lormetazepam are included in the manufacture's kit insert. The cross-reactivity of lorazepam is stated as 90% at a concentration of 50 pg/well and 85% at a concentration of 100 pg/well. The cross-reactivity of lormetazepam is stated as 120% at a concentration of 500 pg/well. (Immunalysis and Corporation, 2011) Each well holds 350 µL so: 500 pg/well = 500 pg/350 µL or 1,429 pg/1000 µL which is 1.43 ng/mL. The conversion of the insert data is shown in Table 12. The results from the kit insert demonstrates cross-reactivity at very low concentrations, likely to be much lower than the limit of detection of the confirmation techniques therefore the immunoassay may give a positive response that is negative on confirmation.

Table 12: Immunalysis® Benzodiazepine ELISA kit insert results and conversion for lorazepam and lormetazepam

Drug	Concentration on insert	% Cross-reactivity	Converted concentration
Lorazepam	50 pg/well	90	0.143 ng/mL
Lorazepam	100 pg/well	85	0.29 ng/mL
Lormetazepam	500 pg/well	120	1.43 ng/mL

2.5.1. Limitations

The limitations of immunoassay must be considered when interpreting these results. The cross-reactivity of a non-targeted antibody can be both a helpful characteristic as well as a problematic one. Immunoassay offers information on the drug group only; benzodiazepines could contain both licit in the form of prescribed medication and illicit compounds in the same sample. Other compounds in the sample may react and cause a false-positive response (e.g. compounds present due to HIV treatment or antidepressant use e.g sertraline). (Saitman, Park and Fitzgerald, 2014) Sample condition especially decomposed post-mortem blood may also have an effect on how well the kit detects any benzodiazepines. Additional biochemical compounds such as enzymes, other proteins and lipids in the decomposed blood can result in false positives or false negatives.

Benzodiazepines are a large class of drugs and a more specific technique such as LC-MS/MS will always be needed in order to identify what specific benzodiazepines and metabolites are in a sample and at what concentrations.

2.5.2. Case study

The following case study demonstrates how designer benzodiazepines were positively detected by ELISA in a real post-mortem case.

This case study describes the death of a 20-year-old male who according to his medical history had a psychotic disorder. He was prescribed lymecycline, fluoxetine, pregabalin and zuclopenthixol. Items found at the scene indicated he had purchased “legal highs” online. The deceased's blood was screened using the Immunalysis® Benzodiazepine ELISA kit and gave a presumptive positive result. The in-house LC-MS/MS confirmation test used includes 10 benzodiazepines drugs/metabolites: diazepam, desmethyldiazepam, oxazepam, temazepam, lorazepam, 7-aminoflunitrazepam, nitrazepam, chlordiazepoxide, phenazepam and etizolam. Etizolam was confirmed at a concentration of 0.011 mg/L (11 ng/mL). A specific LC-MS/MS confirmation test for pyrazolam was also carried out due to suspected pyrazolam tablets found at the scene. Pyrazolam was also found in the blood at a concentration of 0.070 mg/L (70 ng/mL). All of the other benzodiazepines tested were negative. This suggests that the Immunalysis® Benzodiazepine ELISA kit can identify etizolam and/or pyrazolam in post-mortem blood.

2.6. Conclusion

This study determined that the designer benzodiazepines, phenazepam, etizolam, pyrazolam, flubromazepam, diclazepam and delorazepam cross-react with the Immunalysis® Benzodiazepine ELISA kit. When the calibrators were made up in PBS the mean cross-reactivity was 113, 109, 104, 97, 104 and 86% for phenazepam, etizolam, pyrazolam, flubromazepam, diclazepam and delorazepam, respectively. When the calibrators were prepared in blood, the average cross-reactivity was 90, 107, 86, 84, 79 and 80% for phenazepam, etizolam, pyrazolam, flubromazepam, diclazepam and delorazepam, respectively. (O'Connor, Torrance and McKeown, 2016) This study demonstrates that the Immunalysis® Benzodiazepine ELISA kit can be used as a screening technique for the designer benzodiazepines tested. (O'Connor, Torrance and McKeown, 2016) A positive

blood sample at the concentration of 5 ng/mL and above should be detectable however as the application of this study would be in post-mortem blood, sample condition must be considered. As post-mortem blood samples are often decomposed, clotted, fatty or oily the same results may not be achieved and blood in these conditions could not be tested in the same way as in this study.

3. Validation of a LC-MS/MS method for the quantitation of diclazepam, delorazepam, lormetazepam, flubromazepam and pyrazolam in blood

3.1. Introduction

Forensic and clinical toxicologists need reliable analytical methods to ensure the correct interpretation of toxicological results. As discussed in chapter 1, section 1.10, LC-MS(/MS) rather than GC-MS(/MS) is the preferred instrumental technique for benzodiazepine detection. There are many published LC-MS(/MS) methods published for the well-established, prescribed benzodiazepines (Nakamura, 2011; Persona *et al.*, 2015; Dunlop *et al.*, 2017; Montenarh *et al.*, 2014; Edinboro and Poklis, 1994; Marin and McMillin, 2010; Glover and Allen, 2010; Marin *et al.*, 2008; Marin *et al.*, 2012; Ngwa *et al.*, 2007) and these can be adapted to accommodate the new designer benzodiazepines. The challenge for a well-equipped laboratory is the lack of literature pertaining to the new compounds and the lack of available certified reference materials. Without certified reference materials a laboratory cannot be fully confident in the presence of an analyte. The pace of the newly emerging drugs has been rapid compared to the production of reference materials and the requirement for both the parent drug and the metabolites is a further challenge. (Archer, Treble and Williams, 2011) The appearance of a novel drug may be sudden and have a very brief lifetime that presents a commercial challenge to the reference material suppliers; in turn it can be very expensive for laboratories to purchase the required materials if they are available.

Analytical methods must be validated to ensure they are fit for purpose in providing accurate, robust and reproducible data. (International Organization for Standardization, 2017) Unreliable methods could be challenged in court and have legal implications or result in a patient receiving the wrong treatment therefore validation is very important. An interesting example of misleading forensic evidence is the Patricia Stallings case in the USA, she was convicted and sentenced to life in prison for murdering her son by causing him to ingest anti-freeze. Tests appeared to show a high concentration of ethylene glycol in the blood that supported the anti-freeze theory. Patricia's other son then began to demonstrate the same symptoms whilst Patricia had no access to him. He was diagnosed with Methylamalonic Acidemia (MMA), a rare genetic condition that causes the body to produce propionic acid. Ethylene glycol and propionic acid have a very similar chemical formula, which only differ by one carbon atom. Re-

analysis of the deceased's tissue samples showed he also suffered from MMA. Patricia Stallings was exonerated and received several million dollars in compensation from her lawsuits against the hospital and testing laboratory. A professor who became involved with the case sent samples containing methylamalonic acid to several commercial laboratories and around half of them reported the wrong result. (The National Registry of Exonerations, 2012) Instances like the Patricia Stallings case show the gravity of incorrect forensic evidence. The scientific community has produced various articles to guide scientists on validation in an attempt to come to an international consensus. (Scientific Working Group for Forensic Toxicology, 2013; Wille *et al.*, 2011; Booth *et al.*, 2015; Wille *et al.*, 2017)

3.2. Aim

The aim of this work was to validate a quantitative LC-MS/MS method for diclazepam, delorazepam, lormetazepam, pyrazolam and flubromazepam in blood according to the guidelines published in the "Standard Practices for Method Validation in Forensic Toxicology" published by The Scientific Working Group for Forensic Toxicology (SWGTOX) (Scientific Working Group for Forensic Toxicology, 2013) which have subsequently been replaced by the ANSI/ASB Standard 036 for Method Validation in Forensic Toxicology. (LeBeau, 2020) These guidelines were used, as they are specific to the validation of methods used for forensic toxicology and are the product of expert consensus on method validation.

3.3. Materials and Methods

3.3.1. Materials

Diclazepam (1mg/mL in MeOH, >99%), pyrazolam (1mg/mL in ACN, >99%), and flubromazepam (1mg/mL in MeOH, >99%), were purchased from Chiron (Trondheim, Norway). Delorazepam (0.1mg/mL in MeOH, >99%), lormetazepam (1mg/mL in MeOH, >99%), lorazepam-D4 (0.1mg/mL in MeOH, >99%), and diazepam-D5 (0.1mg/mL in MeOH, >99%), were purchased from Sigma Aldrich (Gillingham, UK). DiH2O was prepared in-house using the Purite (Thame, UK) diH2O system. Disodium hydrogen orthophosphate anhydrous (AR Grade) and sodium dihydrogen orthophosphate dihydrate (AR Grade) were purchased from Fisher Scientific (Loughborough, UK). Ammonium acetate (AR grade) was purchased from Sigma Aldrich (Gillingham, UK). Methanol (HPLC grade),

acetonitrile (LC/MS grade), cyclohexane (CP grade), ethyl acetate (Tech grade), ammonia (AR grade, 28%) and formic acid (AR grade, >99%) were all purchased from VWR (Lutterworth, Leicestershire, UK). Expired red blood cells were purchased from the Scottish National Blood Transfusion Service (SNBTS) at Gartnavel Hospital (Glasgow, UK). Human whole blood was purchased from Biological Specialty Corporation (Pennsylvania, USA). The solid phase extraction United Chemical Technologies CleanScreen® DAU (200 mg, 10 mL) (SPE) cartridges used were purchased from Chromatography Direct (Runcorn, UK).

3.3.2. Reagent preparation

The reagents used in the extraction and their preparation is detailed in Table 13. Class A graduated measuring cylinders and volumetric flasks were used to make up the reagents and the contents mixed prior to transferring to glass reagent bottles.

Table 13: Blood method validation reagent preparation

Reagent	Substance 1	Substance 2	Substance 3	Substance 4	Storage
Phosphate buffer (pH 6, 0.1M)	Disodium hydrogen orthophosphate anhydrous *1.7 g*	Sodium dihydrogen orthophosphate dihydrate *12.14 g*	DiH_2O *Up to 1000 mL*	0.1M monobasic/dibasic sodium phosphate (for pH adjustment)	Room temp.
Phosphate buffer (pH 6, 0.1M): DiH_2O (1:2, v:v)	Phosphate buffer (pH 6, 0.1M) *500 mL*	DiH_2O *1000 mL*	-	-	Room temp.
Phosphate buffer: Acetonitrile (80:20, v:v)	Phosphate buffer (pH 6, 0.1M) *160 mL*	Acetonitrile *40 mL*	-	-	Room temp.
Ethyl acetate with 2% ammonium	Ethyl acetate *980 mL*	Ammonia *20 mL*	-	-	Room temp.
Blank blood	Red blood cells *500 mL*	DiH_2O *500 mL*	-	-	Fridge (2-8°C)
Reconstitution solution (50:50, v:v)	Methanol *500 mL*	DiH_2O *500 mL*	-	-	Room temp.
Reconstitution ACN solution (25:75, v:v)	Acetonitrile *50 mL*	DiH_2O *150 mL*	-	-	Room temp.
2M ammonium acetate	Ammonium acetate *15.4 g*	DiH_2O *100 mL*	-	-	Room temp.

Mobile Phase A	DiH_2O *2000 mL*	2M ammonium acetate *2 mL*	Formic acid *2 mL*	-	Room temp.
Mobile Phase B	Methanol *2000 mL*	2M ammonium acetate *2 mL*	Formic acid *2 mL*	-	Room temp.

3.3.3. Calibrator, QC and Internal Standard Preparation

The preparation details for the calibrator stock and working solutions are detailed in Tables 14 and 15. Class A volumetric flasks were used to make up the solutions and vigorously inverted several times to mix. Both stock and working solutions were stored in the freezer (≤-20 °C).

Table 14: Blood method validation calibrator stock preparation

	Drug @ 1 mg/mL ***100 µl added***	**Drug @ 100 µg/mL** ***1 mL added***	**Solvent** ***Up to 10 mL***
Calibrator stock solution @ 10 µg/mL	Diclazepam Lormetazepam Pyrazolam Flubromazepam	Delorazepam	Methanol

Table 15: Blood method validation calibrator working solution preparation

	Substance 1	**Substance 2**
Calibrator working solution @ 1 µg/mL	Calibrator stock solution @ 10 µg/mL *1 mL*	Methanol *Up to 10 mL*

The quality control (QC) stock and working solutions were prepared in the same way as the calibrator solutions at the same concentration, see Tables 14 and 15. The QCs were prepared using reference standards with different lot numbers from the calibrators and were prepared independently to the calibrators by a different analyst in order to avoid preparation errors. The internal standard preparation is shown in Tables 16 and 17. Diazepam-D5 is used as the internal standard for all analytes except lormetazepam which uses lorazepam-D4. When the validation tests were carried out the only internal standard used for all analytes was diazepam-D5. Lorazepam-D4 is used for lormetazepam more recently as it has a closer retention time and is closer structural to lormetazepam. Table 18 displays the preparation of the calibrators and QCs using the working solutions prepared in table 15.

Table 16: Blood method validation internal standard stock preparation

	Drug @ 100 µg/mL ***1 mL added***	**Solvent** ***Up to 10 mL***
Internal standard stock solution @ 10 µg/mL	Diazepam-D5 Lorazepam-D4	Methanol

Table 17: Blood method validation internal standard working solution preparation

Solution	**Substance 1**	**Substance 2**

Internal standard working solution @ 1 µg/mL	Internal standard stock solution @ 10 µg/mL *1 mL*	Methanol *Up to 10 mL*

Table 18: Blood method validation calibrators and QC preparation

Calibrator/QC	Final Concentration (mg/L)	Volume of Working Calibrator/ Spike/QC Solution (1 µg/mL) (µL)
Calibrator 1	0.005	5
Calibrator 2	0.010	10
Calibrator 3	0.020	20
Calibrator 4	0.050	50
Calibrator 5	0.100	100
Calibrator 6	0.200	200
Spike	0.042	42
QC 1	0.015	15
QC 2	0.042	42
QC 3	0.150	150
LOD 3	0.0030	3
LOD 2.5	0.0025	2.5
LOD 2	0.0020	2
ME Low	0.010	10
ME High	0.170	170
Carry-over	0.500	500

3.3.4. LC-MS/MS Instrumentation and parameters

The analysis was carried out using an Agilent 1200 LC system coupled to an AB Sciex 3200 QTRAP MS. The software utilised was Analyst® 1.7. The column oven was set at 40 °C and was fitted with a Phenomenex Gemini C18 column (150 mm x 2.0 mm, 5 µm) which had a pre column SecurityGuard™ cartridge (4.0 mm x 2.0 mm) in place. A reversed phase isocratic system consisting of two mobile phases; 2 mM ammonium acetate and 0.1% formic acid in diH_2O water (mobile phase A) and 2 mM ammonium acetate and 0.1% formic acid in methanol (mobile phase B) was used. The isocratic system was maintained at 40/60% A/B with a total runtime of 7 mins. The injection volume was 10 µL.

Positive electrospray ionisation was utilised and the ion source temperature was maintained at 350°C. Nitrogen was used as the source and collision gas. The collision gas (CAD) was set at 4 and the ion source gas 1 and 2 were set at 30 psi. Multiple reaction monitoring (MRM) mode was used to detect the analytes see Table 19.

The MRM transitions in Table 19 were chosen as they displayed the greatest abundance when subjected to post-column infusion. Diazepam-D5 was the internal standard for all analytes except lorametazepam, which used lorazepam-D4.

Table 19: Blood method analytes and MS parameters

Name	Q1	Q3	DP	EP	CEP	CE	CXP
Diclazepam QT	319.1	227.2	71	10.5	16	43	6
Diclazepam QL	319.1	154.1	71	10.5	16	39	6
Delorazepam QT	305.1	140.0	66	10.5	12	41	6
Delorazepam_QL	305.1	206.1	66	10.5	12	49	6
Lormetazepam QT	335.1	289.1	46	10.5	16	31	6
Lormetazepam QL	335.1	177.0	46	10.5	16	55	6
Flubromazepam QT	333.0	226.2	71	10.5	14	41	6
Flubromazepam QL	333.0	184.0	71	10.5	14	43	6
Pyrazolam QT	354.1	206.2	61	11.0	16	43	6
Pyrazolam QL	354.1	167.0	61	11.0	16	45	6
Diazepam-D5	290.1	198.1	66	10.5	14	45	4
Lorazepam-D4	325.1	279.2	51	10.0	14	33	6

3.3.5. Sample extraction.

The extraction was carried out by SPE, Figure 21 shows a flowchart outlining the SPE method used. SPE was chosen as the extraction technique for this method as the samples tested were post-mortem blood, which is often decomposed or fatty, SPE provides a clean extract. LLE would produce a less clean extract, which in turn would result in an increased need for instrument maintenance.

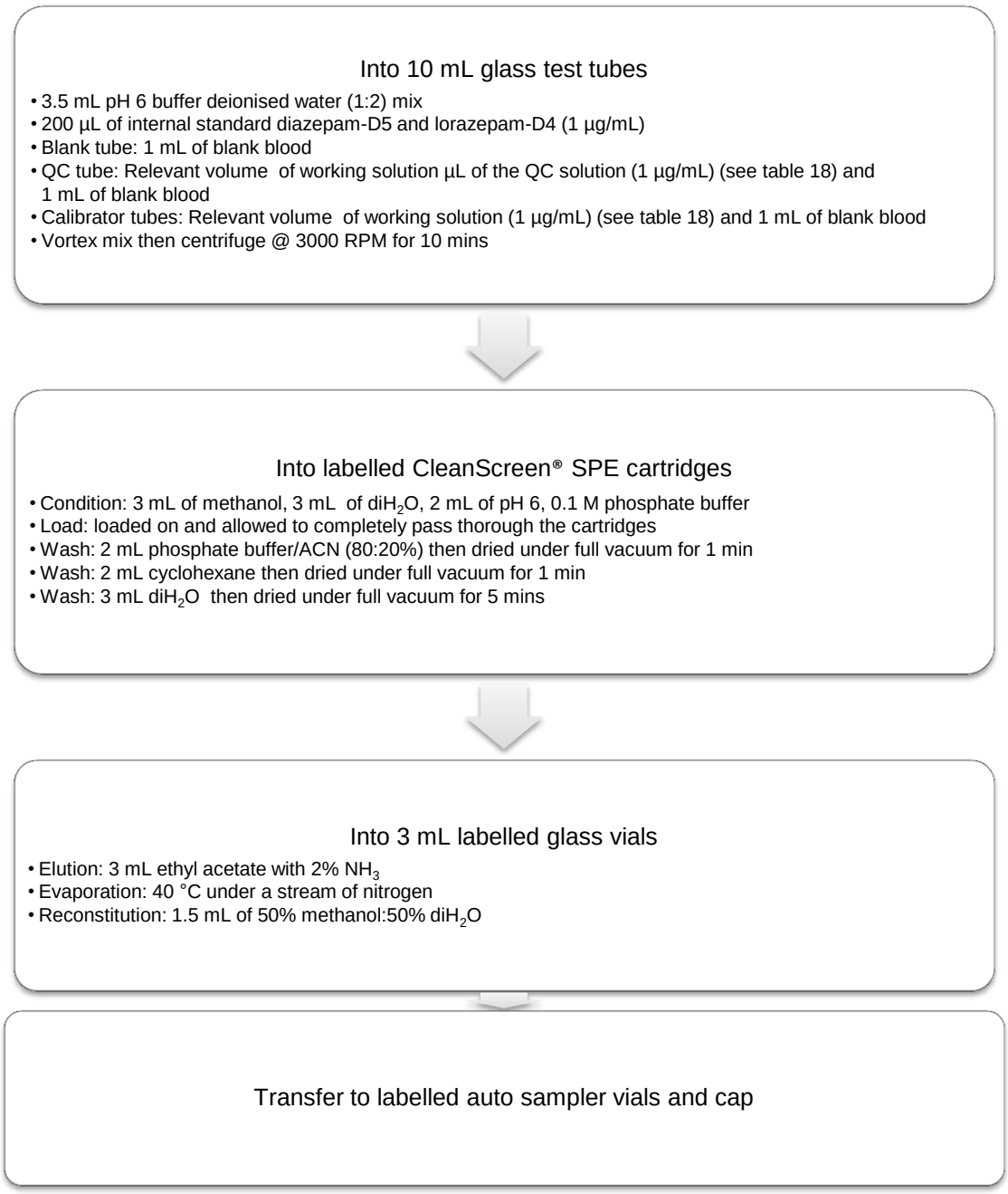

Figure 21: Blood method - SPE procedure

3.3.6. Data handling and statistical analysis

Once results had been generated in the Analyst software, they were copied onto a Microsoft® Excel® (version 14.7.3) spreadsheet and batch checked by a second toxicologist before any calculations were performed. The results copied include the peak areas of the analyte and the internal standard, the calculated values of the calibration standards and QCs in mg/L and the datapath information. Microsoft® Excel® was then used to calculate the mean, standard deviation, %CV, %ME,

%PE and %RE where necessary. A second toxicologist, as part of the batch checking, also checked the formula used in the spreadsheets for each calculation. Line graphs were generated in the spreadsheet to track autosampler stabilty. The Statsplus (AnalystSoft™, version 7.7.31) add on for Microsoft® Excel® was used to generate residual plots to investigate linearity. The spreadsheets were saved on a secure drive to protect the data.

3.4. Method validation experimental

3.4.1. Selectivity

Selectivity has been described, as "the ability to unambiguously assess the analyte of interest while in the presence of all expected components, which may consist of degradants, excipients/sample matrix, and sample blank peaks." (Scientific Working Group for Forensic Toxicology, 2013; Tiwari and Tiwari, 2010) Endogenous substances such as proteins, salts, lipids and small molecules can be a common source of interference. There should be no interfering signals at the retention times of interest for the drugs/metabolites.

A selectivity experiment was carried out using 10 different sources of drug free whole blood extracted in singlicate within a single batch to determine if endogenous interference is an issue.

3.4.2. Specificity

Exogenous interference from other drugs and metabolites was evaluated by injecting 31 analytes, in triplicate, unextracted in reconstitution solution at a concentration of 0.5 mg/L. The 31 drugs and metabolites are detailed in Table 20. These drugs were selected, as they are common drugs encountered in forensic toxicology. They are not all benzodiazepines or structurally similar to benzodiazepines and were chosen for their commonality in case samples not their structure. These drugs were in 3 individual solutions, solution 1 contained all the drugs from chlorpromazine to amitriptyline, solution 2 contained the benzodiazepines from oxazepam to nitrazepam and solution 3 was a drugs of abuse mix containing all the drugs from cocaine to 6-MAM in Table 20.

Table 20: Blood method validation - Drugs used to evaluate specificity

Chlorpromazine	Phenazepam

Tramadol	Etizolam
Diphenhydramine	Lorazepam
Lignocaine	Nitrazepam
Methadone	Cocaine
Citalopram	Benzoylecognine
Chlorpheniramine	Amphetamine
Mirtazapine	Methamphetamine
Diltiazem	MDMA
Zolpidem	MDA
Amitriptyline	MDEA
Oxazepam	Morphine
Chlordiazepoxide	Codeine
Diazepam	Dihydrocodeine
Desmethyldiazepam	6-MAM
Temazepam	

3.4.3. Linearity

Linearity is crucial as it "assesses the ability of the method to obtain test results that are directly proportional to the concentration of the analyte in the sample." (Tiwari and Tiwari, 2010)

For the calibrators, blank blood was spiked with each analyte at six concentrations from 0.005 to 0.20 mg/L, in singlicate, within ten batches over ten months; these were used to evaluate linearity. A blank (with internal standard) was extracted with each calibration curve. The concentration range was from 0.005 – 0.2 mg/L; six calibrators at the concentrations of 0.005, 0.010, 0.020, 0.050, 0.10 and 0.20 mg/L make up the calibration curves, see Table 18 for preparation details. Calibration curves were generated using the Analyst 1.7 software. All calibrators within each curve must have an accuracy within ±20% of the target value and the curves must have a correlation coefficient (r) greater than 0.99 (r>0.99).

The standard deviation (SD) and coefficient of variation (%CV) were calculated for the ten calibration curves using equations 2 and 3.

Equation 2: Standard deviation

$$SD = \sqrt{\frac{\sum(x-\overline{x})^2}{(n-1)}}$$

Where x is the value, $\overline{x}$ is the mean and n is the sample size.

Equation 3: %Coefficient of variation

$$\%CV = SD/\overline{x} \text{ X } 100$$

Where $\overline{x}$ is the mean.

The precision of the lower limit of quantitation (LLOQ) and the upper limit of quantitation (ULOQ) will be evaluated. The inter-day precision of the LLOQ and ULOQ was calculated using %CV, see equation 3, for the ten calibration curves.

3.4.4. Limit of detection

The limit of detection (LOD) is the "lowest concentration of an analyte in a sample which can be detected but not necessarily quantified as an exact value." (Tiwari and Tiwari, 2010)

LOD can be determined by measuring the signal-to-noise ratio of the peak. As there is instrumental noise present even in a drug free injection, the s/n ratio was used to determine the LOD in this study as this approach takes that noise into consideration, calculating LOD by equation does not account for background noise. The Analyst ® software is able to assist with this as it allows the operator to highlight the peak (signal) and a suitable area of noise close to the peak of interest in the baseline. It then calculates the signal-to-noise ratio and displays this on the peak.

LOD for each analyte was evaluated. Extracted concentrations of 0.003, 0.0025 and 0.002 mg/L (see Table 18 for preparation details) were tested in duplicate. The signal-to-noise ratio should be ≥3 for the analyte to be considered detectable. Retention time for each analyte should be consistent.

3.4.5. Precision and bias

Precision is the degree of scatter or closeness of agreement in a series of measurements. The %CV (equation 3) is used to demonstrate precision. For the %CV to be considered acceptable it should be less than 20%. Bias is expressed as the percentage accuracy, which shows deviation from the target value. Bias demonstrates the difference between the test results and the target value. The acceptable range is ±20%).

Three different QC concentrations (0.015, 0.042 and 0.150 mg/L), see Table 18 for preparation details, were each extracted five times and run within one batch on the same day for intra-day bias and precision. Three different QC concentrations

(0.015, 0.042 and 0.150 mg/L) were each extracted five times and run over five different batches run on five different days for inter-day bias and precision.

3.4.6. Matrix effects, recovery and process efficiency

Interfering compounds within the sample matrix or reagents such as buffers can cause matrix effects (ME). Sample nature can dictate the occurrence of matrix effects, which makes them very unpredictable. Using an LC-MS method requires assessment of the matrix effects phenomenon, due to the techniques susceptibility to ion enhancement or suppression, which can lead to inaccurate quantitative results.

Ion suppression occurs when less volatile compounds change the efficiency of the droplet formation, which leads to less of the charged ion in the gas phase that reaches the detector. (Annesley, 2003) The mechanism responsible for ion enhancement is still not fully understood but it leads to an increase in the formation of ions. The ME can be expressed as %accuracy. The %ME range should fall within ±25% to be considered acceptable. Ion suppression will be shown as <100% and ion enhancement as >100%. The precision (%CV) should not exceed 15% according to SWGTOX. (Scientific Working Group for Forensic Toxicology, 2013)

Recovery (RE) can also be assessed with the same set of experiments used to evaluate ME. Recovery is defined as the percentage of analyte extracted from the sample matrix at the end of the analysis. The importance of recovery is argued; with some authors stating that providing other parameters such LOD is acceptable there is no requirement for a high percentage of recovery. (Tiwari and Tiwari, 2010; Peters, Drummer and Musshoff, 2007) It is recommended that recovery should be at least 50%.

Process efficiency (PE) represents the combination of ME and RE to give an overall assessment of the difference the matrix has induced on each analyte during the complete extraction process, compared to the analyte in reconstitution solution.

For two concentrations investigated, ME, RE and PE were calculated using the post-extraction addition approach detailed by Matuszewski *et al.* (Matuszewski, Constanzer and Chavez-Eng, 2003)

Drug-free (blank) whole blood from 10 different sources was used for the ME, RE

and PE experiments. The analytes, diclazepam, delorazepam, lormetazepam, pyrazolam and flubromazepam, at two different concentrations (ME Low 0.010 and ME High 0.17 mg/L, see Table 18), were evaluated. The ME, RE and PE experiments were performed as follows:

- Set A: neat unextracted analyte mix and internal standard in reconstitution solution was prepared six times.
- Set B: blank blood from 10 different sources were spiked with the analyte mix and internal standard in reconstitution solution after SPE. Set B was prepared in duplicate.
- Set C: blank blood from 10 different sources were spiked with analyte mix and internal standard prior to SPE. Set C was prepared in singlicate.

The percentage matrix effects (%ME) were calculated using equation 4.

Equation 4: %Matrix effects

$$\%ME = \left(\frac{B}{A}\right) \text{ X } 100$$

Where

A = Mean peak area of neat unextracted analyte

B = Mean peak area of post-extraction spiked analyte

The percentage recovery (%RE) was calculated using equation 5.

Equation 5: %Recovery

$$\%RE = \left(\frac{C}{B}\right) \text{ X } 100$$

Where

B = Mean peak area of post extraction spiked analyte

C = Mean peak area of extracted analyte

The percentage process efficiency (%PE) was calculated using equation 6.

Equation 6: %Process efficiency

$$\%PE = \left(\frac{C}{A}\right) \text{ X } 100$$

Where

A = Mean peak area of neat unextracted analytes

C = Mean peak area of extracted analyte

3.4.7. Carryover

Examining injections of drug-free reconstitution solution, which are injected directly following a high concentration for analyte peaks, provides an assessment of carryover. There should be no signal at or near the retention time of the analytes of interest. An extracted concentration of 0.5 mg/L of diclazepam, delorazepam, lormetazepam, pyrazolam and flubromazepam, was injected in triplicate followed, by three injections of drug-free reconstitution solution (50:50% methanol:diH2O). The concentration 0.5 mg/L was chosen, as this is more than double the ULOQ.

3.4.8. Autosampler stability

Stability can be defined as “the chemical stability of an analyte in a given matrix under specific conditions for given time intervals.” (Tiwari and Tiwari, 2010) It is important to evaluate stability to address the circumstances encountered in normal laboratory situations. In a busy laboratory environment vials may sit on the autosampler for several hours before the run is complete.

Blank blood was spiked at the high QC (0.15 mg/L) and the low QC (0.015 mg/L) concentration in triplicate and extracted using the SPE procedure detailed in Figure 21. These extracts were then run on the method detailed in section 3.3.4 to determine a time zero response (t0), the same extracts were then injected at three hour intervals up to 48 hours. The autosampler was not temperature controlled. The laboratory was temperature controlled between 16°C and 24°C during the experiment. This reflects the normal temperature range of the laboratory in which real life samples are analysed.

3.5. Method Validation Results and Discussion

3.5.1. Selectivity

An absence of interfering signals at or near the retention times of the analytes of interest, diclazepam, delorazepam, lormetazepam, flubromazepam and pyrazolam, was demonstrated by this experiment, there were no viable peaks detected for the ten sources of blank blood tested. Figures 22 and 23 display example chromatograms with and without internal standard respectively, from one of the sources of blank blood tested; the analyte retention times have been added to show each analytes position and the lack of interference in the area of interest. The green and blue peaks are lorazepam-D4 and diazepam-D5 respectively.

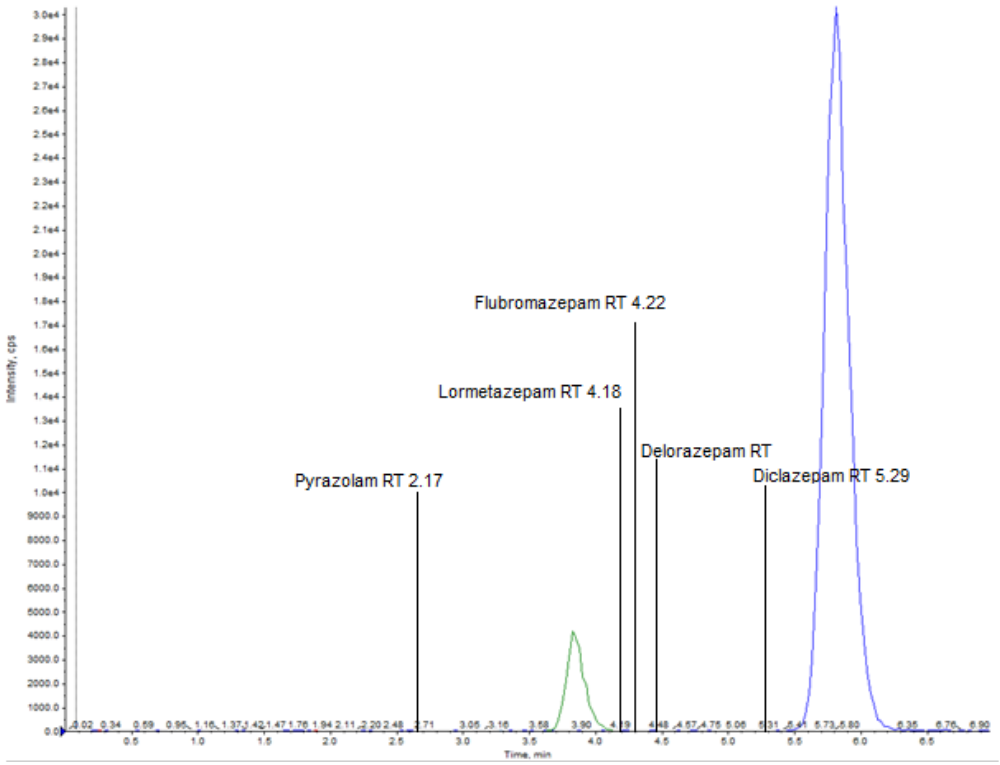

Figure 22: Example chromatogram of method selectivity, with internal standard, lack of interfering signal at the retention time of the analytes of interest.

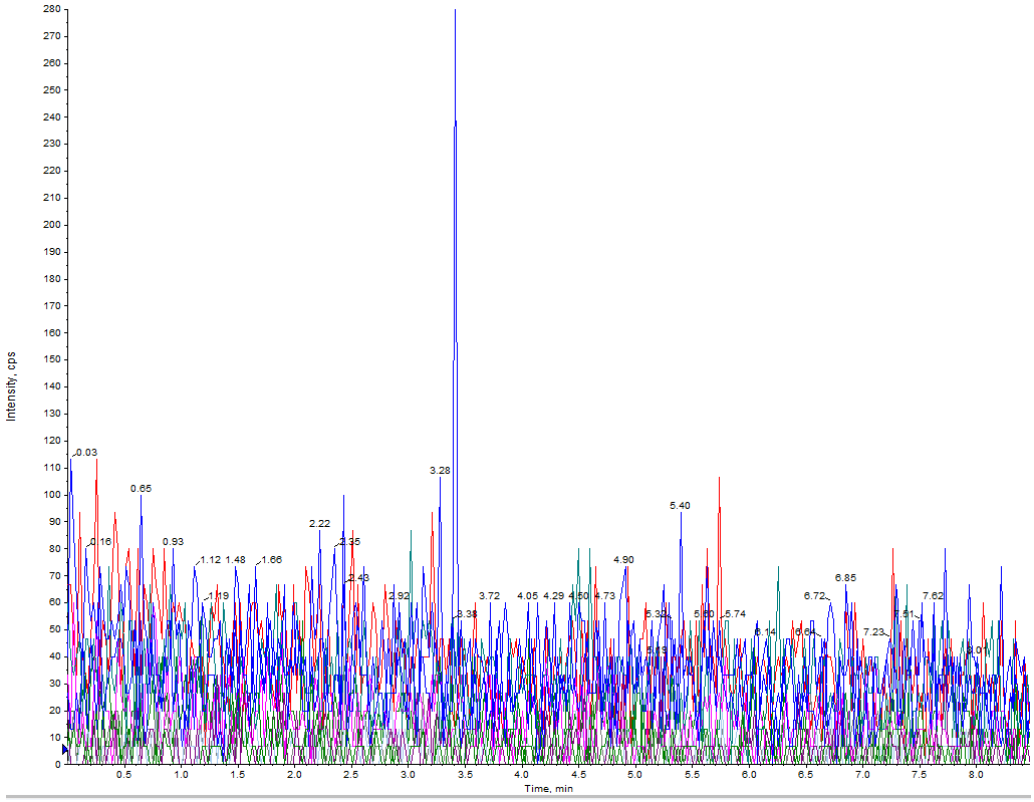

Figure 23: Example of method selectivity, without internal standard, lack of interfering signal at the retention time of the analytes of interest.

3.5.2. Specificity

No interfering peaks were detected from any of the 31 drugs/metabolites at the retention times of interest of any of the analytes tested for in this method. Figure 24 shows the blank signal, which was obtained after the injection of solution 3 (see 3.4.2) which included cocaine, benzoylecognine, amphetamine, methamphetamine, MDMA, MDA, MDEA, morphine, codeine, dihydrocodeine and 6-MAM. All drugs within Table 20 gave no signal, similar to the example shown in Figure 24.

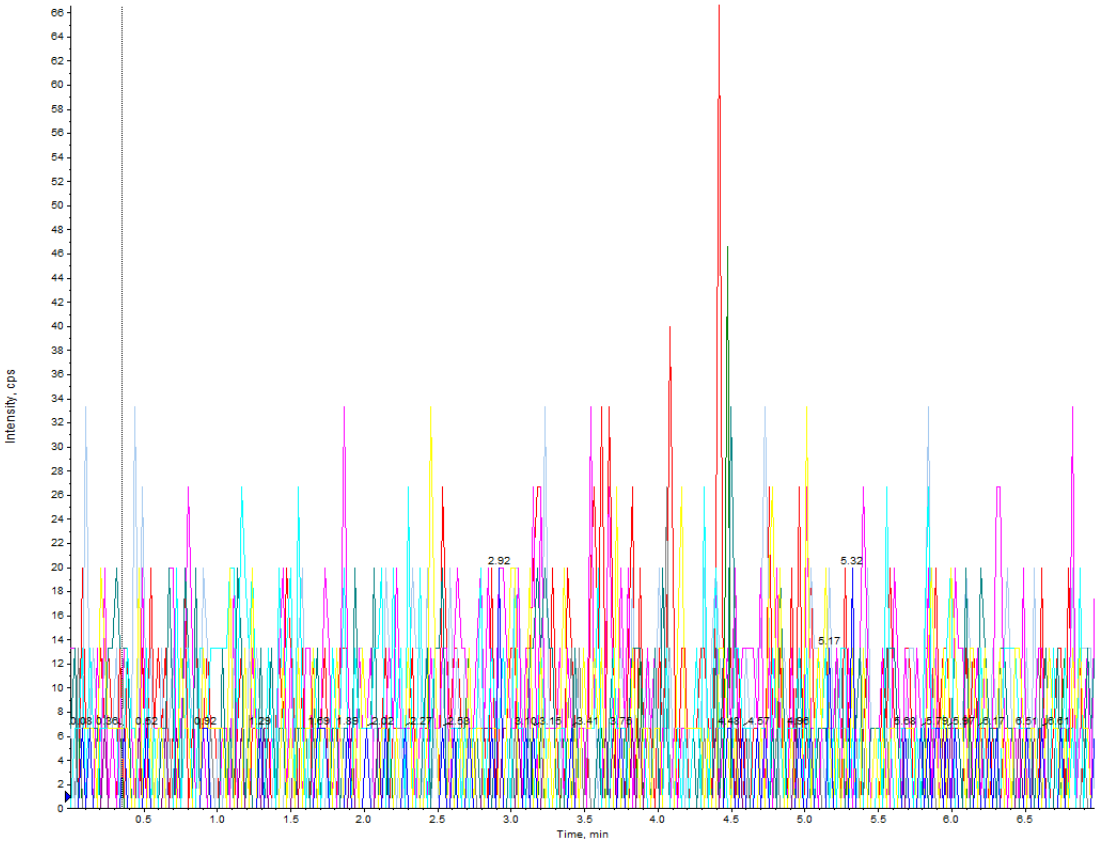

Figure 24: Example chromatogram of method specificity, from other drugs/metabolites, shown by the lack of signal at the analytes of interest retention time.

3.5.3. Linearity

Linearity was tested with ten calibration curves which where ran over a ten-month period. The linearity data for all 5 analytes is displayed in Table 21. All analytes displayed good linearity with 1/x weighted linear regression over the calibration range 0.005 to 0.20 mg/L, with all curves r>0.99. All calibrators were within the ±20% accuracy criterion. 1/x weighting was chosen to minimise the distribution of

variance across the calibration range, larger deviations present in the higher concentrations will influence the curve more leading to impaired accuracy on the at the lower concentrations. To balance this out weighting is applied, when 1/x weighting is applied the slope more closely approximates the majority of calibrator points. Figure 25 gives example calibration curves for diclazepam, delorazepam, lormetazepam, flubromazepam and pyrazolam. The residuals from five calibration curves were plotted for each analyte (as per SWGTOX recommendation), see Figure 26. The residuals represent the difference in observed value from the predicted value. These values can be positive or negative depending on if the observed value is more or less than the predicted value. A residual plot with randomly distributed positive and negative values around the 0 axis with no defined trend demonstrates a linear model is appropriate. (Polettini, 2006) There is no convincing pattern in the residual plots for diclazepam, delorazepam, lormetazepam, and pyrazolam, demonstrating linearity is achieved. The range of variances increases with the concentration confirming a weighted calibration is required which was applied. (Polettini, 2006) Flubromazepam appears to be more positively distributed and therefore a quadratic fit may be more appropriate however the calibration curves produced have all been linear with a r>0.99. It is recognised that when calibration points are displaying accuracy within the acceptable limits then an alternative fit is not required and deviations can be overlooked. (Peters and Maurer, 2002)

Table 21: Blood method validation linearity data

Analyte	**Mean r (n=10)**	**SD**	**%CV**	**Calibrator % accuracy range**
Diclazepam	0.9994	0.0005	0.0539	93-108
Delorazepam	0.9992	0.0011	0.1130	84-114
Lormetazepam	0.9981	0.0029	0.2874	90-114
Pyrazolam	0.9963	0.0024	0.2362	81-112
Flubromazepam	0.9990	0.0007	0.0686	82-116

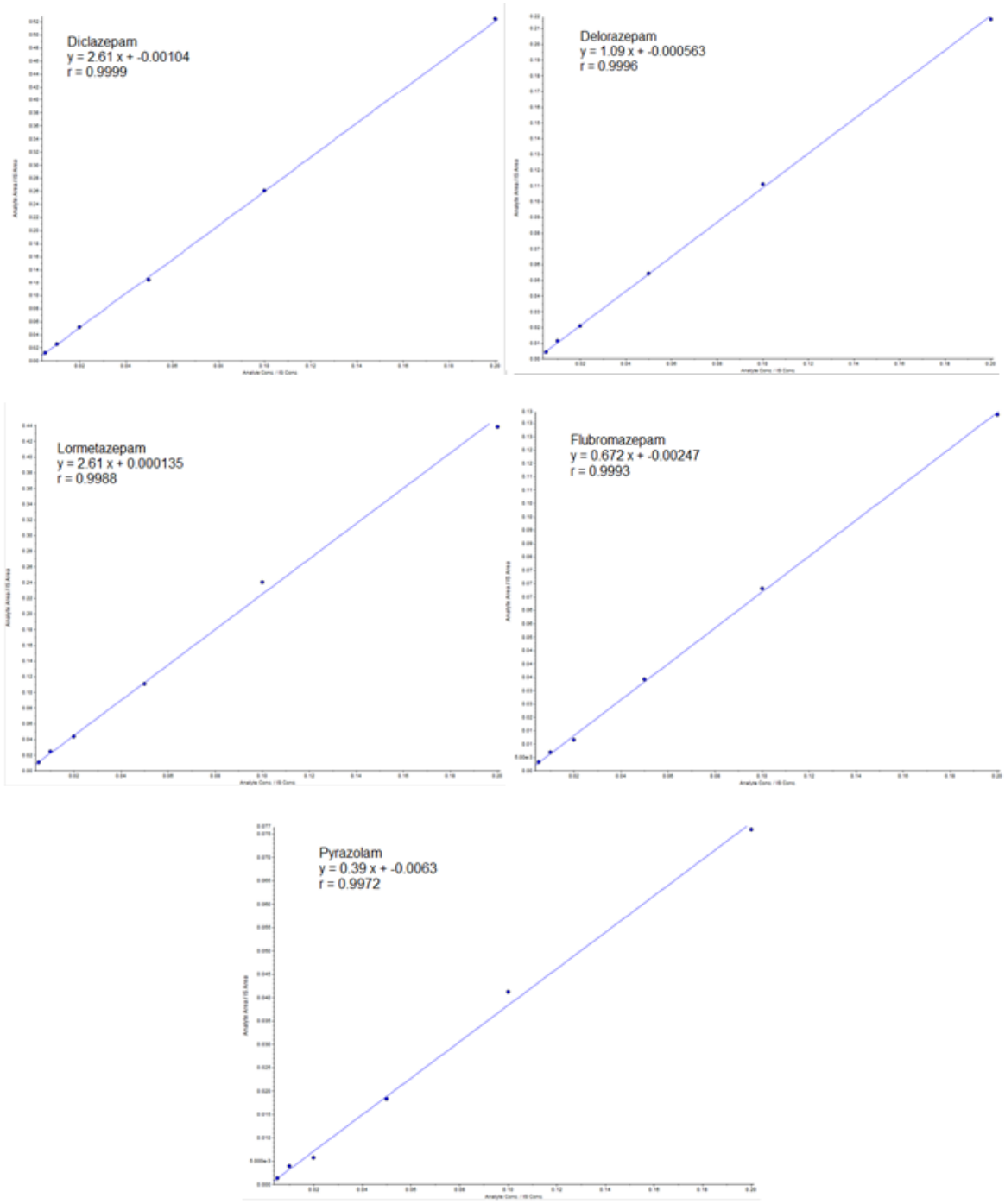

Figure 25: Example calibration curves for diclazepam, delorazepam, lormetazepam, flubromazepam and pyrazolam.

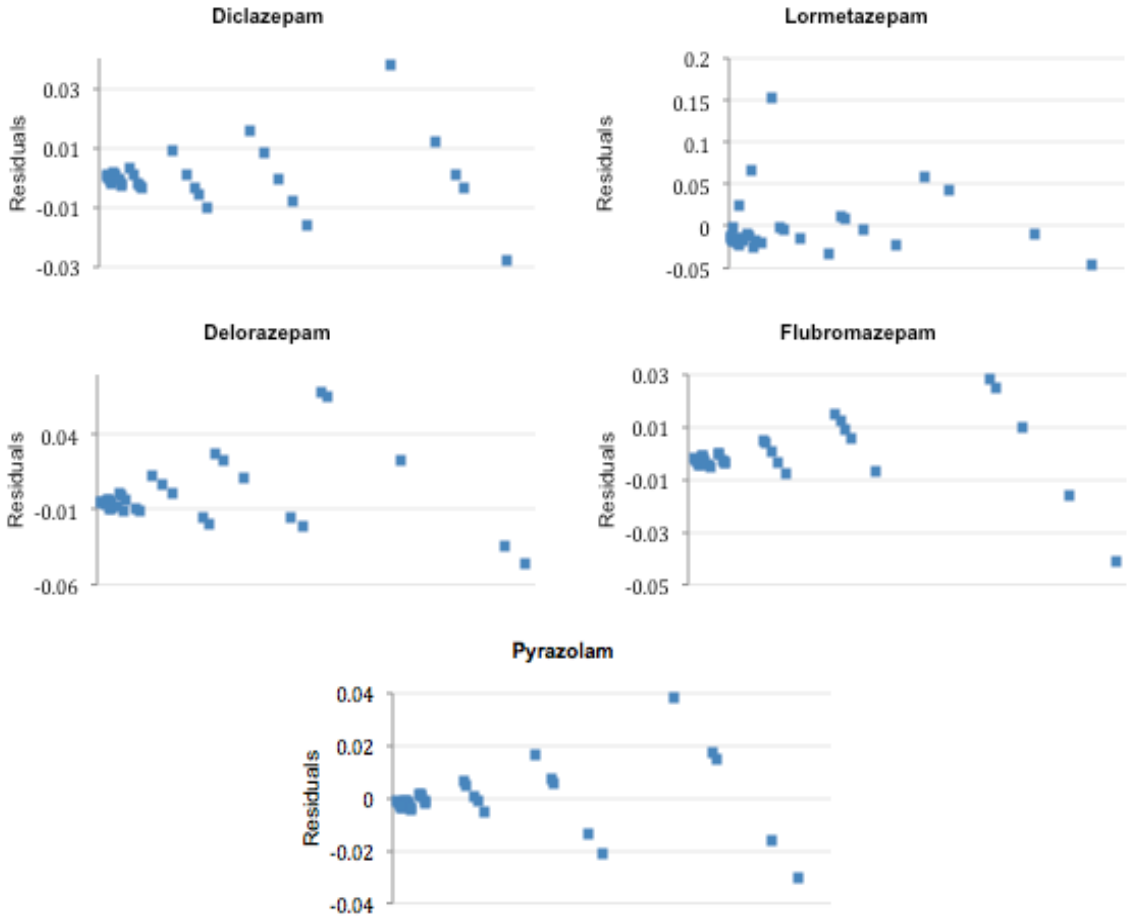

Figure 26: Residual plots for diclazepam, delorazepam, lormetazepam, flubromazepam and pyrazolam

The inter-day precision of the LLOQ and ULOQ was calculated in order to evaluate the low and high point of the calibration curve for all analytes. The inter-day %CV met the acceptance criterion of <20% for diclazepam (<1.5), delorazepam (<9.5), lormetazepam (11.2), flubromazepam (<11.6%) and pyrazolam (<17.2%) for both the LLOQ and ULOQ showing that both high and low points are precise for this method. Diclazepam LLOQ data demonstrates good precision giving a SD and %CV of 0 to 3 decimal places. The %bias was also calculated and demonstrated good accuracy for the LLOQ. Table 22 shows the LLOQ mean concentration, SD and inter-day precision and bias.

Table 22: Blood method validation LLOQ (0.005 mg/L) inter-day precision and bias data

	Diclazepam	Delorazepam	Lormetazepam	Flubromazepam	Pyrazolam
Mean concentration	0.005	0.005	0.005	0.005	0.005
SD	0.000	0.000	0.001	0.001	0.001
%CV	0.0	9.4	11	12	17
%Bias	0	0	-2	-4	0

Table 23 shows the data used to calculate the ULOQ precision and bias. The precision and bias for the ULOQ for all analytes is particularly good at <5% and <2%, respectively.

Table 23: Blood method validation ULOQ (0.20 mg/L) inter-day precision and bias data

	Diclazepam	Delorazepam	Lormetazepam	Flubromazepam	Pyrazolam
Mean concentration	0.197	0.198	0.199	0.199	0.200
SD	0.003	0.004	0.006	0.004	0.010
%CV	1.4	2.1	3.2	2.1	5.0
%Bias	3	1	1	1	2

3.5.4. Limit of detection

The limit of detection (LOD) was determined by evaluating the signal-to-noise ratios for the analytes of interest. LOD is an approximation of the lowest concentration detectable by the method and can change depending on the set of experimental data used, laboratory conditions and how clean the instrument is. Since this is just an approximation of detectability the S/N ratio is not used to determine LOQ. S/N uses the baseline as a blank in which to compare to the signal generated by the analyte, a blank without the analyte cannot be used accurately as the noise is dependent on the signal magnitude. (Desimoni and Brunetti, 2015) A signal-to-noise ratio ≥3 at the correct retention is considered to be a detectable peak. Using the baseline means instrumental noise is considered but also can widely vary between injections. The S/N ratio alone is not the only aspect considered when using this technique for LOD determination; the peaks must also be visually inspected to ensure good Gaussian peak shapes. Diclazepam, delorazepam, lormetazepam, flubromazepam and pyrazolam all had signal-to-noise ratios >3 for all the concentrations tested therefore the LOD for all five drugs was determined as 0.002 mg/L (2 ng/mL). Flubromazepam gave the lowest signal-to-noise ratio across the three concentrations. Table 24 displays the signal-to-noise ratio results for all analytes at the three concentrations tested.

Table 24: Blood method - Limit of detection (LOD) data

Analyte	Signal-to-noise ratio					
	LOD 3		LOD 2.5		LOD 2	
	QT	QL	QT	QL	QT	QL
Diclazepam	15.2	26.5	15.2	71.5	13.5	44.5
Delorazepam	53.5	33.5	53.5	4.5	17.5	8.5
Lormetazepam	26.2	7.0	26.2	9.8	12.8	6.5
Pyrazolam	12.0	15.0	12.0	11.0	6.5	12.5
Flubromazepam	6.0	16.0	6.0	24.5	4.5	14.5

3.5.5. Precision and bias

The results for the intra- and inter-day precision and bias data are shown in Tables 25 and 26. The precision evaluated by the %CV was within the acceptance criterion of <20% for diclazepam, delorazepam, lormetazepam, pyrazolam and flubromazepam for all three QC concentrations for both intra- and inter-day batches. The bias evaluated by %bias was within the acceptance criterion of ±20% (accuracy of 80-120%) for diclazepam, delorazepam, lormetazepam, pyrazolam and flubromazepam for all three concentrations for both intra- and inter-day batches.

Table 25: Blood method validation intra-day precision and bias

	Analyte				
	Diclazepam	Delorazepam	Lormetazepam	Flubromazepam	Pyrazolam
QC 1	**0.015 mg/L**				
Mean (n = 5)	0.016	0.016	0.015	0.016	0.014 (n = 4)
SD	0.001	0.001	0.002	0.000	0.002
%CV	3.1	9.3	11	2.5	13
%Bias	10	4	-1	5	-4
QC 2	**0.042 mg/L**				
Mean (n = 5)	0.043	0.041	0.043	0.043	0.050
SD	0.002	0.002	0.002	0.002	0.001
%CV	3.7	4.7	4.3	4.4	3.1
%Bias	2	-3	3	3	18
QC 3	**0.150 mg/L**				
Mean (n = 5)	0.152	0.146	0.151	0.160	0.179 (n = 4)
SD	0.005	0.002	0.007	0.003	0.022
%CV	3.0	1.7	4.7	2.1	12
%Bias	2	--3	0	7	19

Table 26: Blood Validation Method Inter-day precision and bias

	Analyte				
	Diclazepam	**Delorazepam**	**Lormetazepam**	**Flubromazepam**	**Pyrazolam**
QC 1	**0.015 mg/L**				
Mean (n = 5)	0.015	0.014	0.014	0.016	0.014
Low	0.012	0.008	0.010	0.013	0.007
High	0.017	0.017	0.017	0.019	0.020
SD	0.001	0.002	0.002	0.001	0.003
%CV	7.9	17	13	8.4	20
%Bias	2	-6	-5	8	-6
QC 2	**0.042 mg/L**				
Mean (n = 5)	0.044	0.041	0.041	0.048	0.046
Low	0.038	0.030	0.031	0.037	0.039
High	0.052	0.047	0.047	0.057	0.056
SD	0.004	0.005	0.005	0.005	0.007
%CV	8.8	12	11	11	14
%Bias	5	-2	-2	13	9
QC 3	**0.150 mg/L**				
Mean (n = 5)	0.159	0.152	0.159	0.177	0.179
Low	0.135	0.114	0.113	0.139	0.115
High	0.180	0.173	0.191	0.216	0.221
SD	0.011	0.016	0.022	0.021	0.034
%CV	7.1	11	14	12	19
%Bias	6	1	6	18	19

3.5.6. Matrix effects, recovery and process efficiency

The matrix effects for diclazepam, delorazepam, lormetazepam, pyrazolam and flubromazepam are shown for the low concentration (0.010 mg/L) in Table 27 and the high concentration (0.17 mg/L) in Table 28.

Table 27: Blood method validation - ME results at 0.010 mg/L

	%ME (n=2) 0.010 mg/L				
	Diclazepam QT	**Delorazepam QT**	**Lormetazepam QT**	**Pyrazolam QT**	**Flubromazepam QT**
Overall Mean %ME (n=10)	-14	-19	-21	-10	-14
Overall ME %CV	7.5	7.6	7.4	8.5	8.8

The mean %ME for diclazepam, delorazepam, lormetazepam, pyrazolam and flubromazepam were -14, -19, -21, -10 and -14% respectively at the low concentration tested. All mean results were within the ±25% criterion for %ME and <15% for precision.

Table 28: Blood method validation - ME results at 0.170 mg/L

	%ME (n=2) 0.170 mg/L				
	Diclazepam QT	**Delorazepam QT**	**Lormetazepam QT**	**Pyrazolam QT**	**Flubromazepam QT**
Overall Mean %ME (n=10)	16	14	12	9	21
Overall ME %CV	1.2	2.0	2.0	2.6	1.8

The mean %ME for diclazepam, delorazepam, lormetazepam, flubromazepam and pyrazolam were 16, 14, 12, 21 and 9% respectively at the high concentration tested. All mean results were within the ±25% criterion and the precision well under the <15% criteria.

Table 29: Blood method validation - ME internal standard results

	Diazepam-D5 %ME (n=2)	
	(0.010 mg/L)	**(0.170 mg/L)**
Overall Mean %ME (n=10)	-6	6
Overall ME %CV	3.5	2.4

The %ME for the internal standard is shown in Table 29; there is no significant ME evident for the internal standard.

The %RE and %PE for diclazepam, delorazepam, lormetazepam, pyrazolam and flubromazepam are shown for the low concentration (0.010 mg/L) in Table 30 and the high concentration (0.17 mg/L) in Table 31.

Table 30: Blood method validation - RE and PE results at 0.010 mg/L

Drug QT (0.010 mg/L)	%RE mean (%CV) (n=10)	%PE mean (%CV) (n=10)
Diclazepam	57 (10)	49 (7.7)
Delorazepam	64 (9.0)	52 (9.2)
Lormetazepam	42 (9.9)	33 (10)
Flubromazepam	31 (18)	26 (16)
Pyrazolam	28 (18)	25 (17)

Table 31: Blood method validation - RE and PE results 0.17 mg/L

Drug QT (0.017 mg/L)	%RE mean (%CV) (n=10)	%PE mean (%CV) (n=10)
Diclazepam	64 (6.7)	75 (7.4)
Delorazepam	63 (6.8)	73 (7.9)
Lormetazepam	45 (7.6)	50 (9.0)
Flubromazepam	43 (12)	52 (13)
Pyrazolam	27 (7.6)	30 (9.5)

Table 32 Blood method validation- RE and PE internal standard data

Drug	%RE mean (%CV) (n=10)	%PE mean (%CV) (n=10)
Diazepam-D5	17 (3.1)	16 (2.8)

The RE and PE results for diclazepam and delorazepam are acceptable and around 50% or above for both concentrations tested. Lormetazepam was below 50% for RE and PE but over 40% for RE for both concentrations, this is considered suboptimal but should not have a deleterious effect. Flubromazepam has sufficient PE and above 40% RE at the high concentration tested but under 40% for RE and PE at the low concentration tested, therefore the accuracy at the lower end of the calibration curve may not be as good as at the high end; however

the LLOQ shows good precision and bias (see Table 22) and the analyte is detectable down to 0.002 mg/L. The RE and PE for pyrazolam at both concentrations is poor at 30% and under however the LLOQ shows good precision and bias (see Table 22) and the analyte is detectable down to 0.002 mg/L. This suggests an issue with this extraction for pyrazolam. One possibility is that pyrazolam is less soluble in methanol than it is in acetonitrile (pyrazolam is purchased in acetonitrile) and therefore does not fully dissolve at the reconstitution step or there is a stability issue with pyrazolam in the combined methanolic working solution. The ME for all analytes was acceptable and although there was low RE and PE for pyrazolam and flubromazepam the precision and bias were acceptable and an acceptable LOD down to 0.002 mg/L was achieved for both.

The mean PE and RE for the internal standard was suboptimal as it is low at 16% and 17%, see Table 32 however it is consistent across all ten sources and the peak areas are sufficiently strong at 1,000,000 cps or above.

3.5.7. Carryover

Carryover is assessed by the absence or presence of peaks in the injections of drug-free reconstitution solution directly following injections of a high concentration of the analytes of interest. There were no peaks in the drug-free reconstitution solution injection that followed three injections of 0.50 mg/L of diclazepam, delorazepam, lormetazepam, pyrazolam and flubromazepam, see Figure 27.

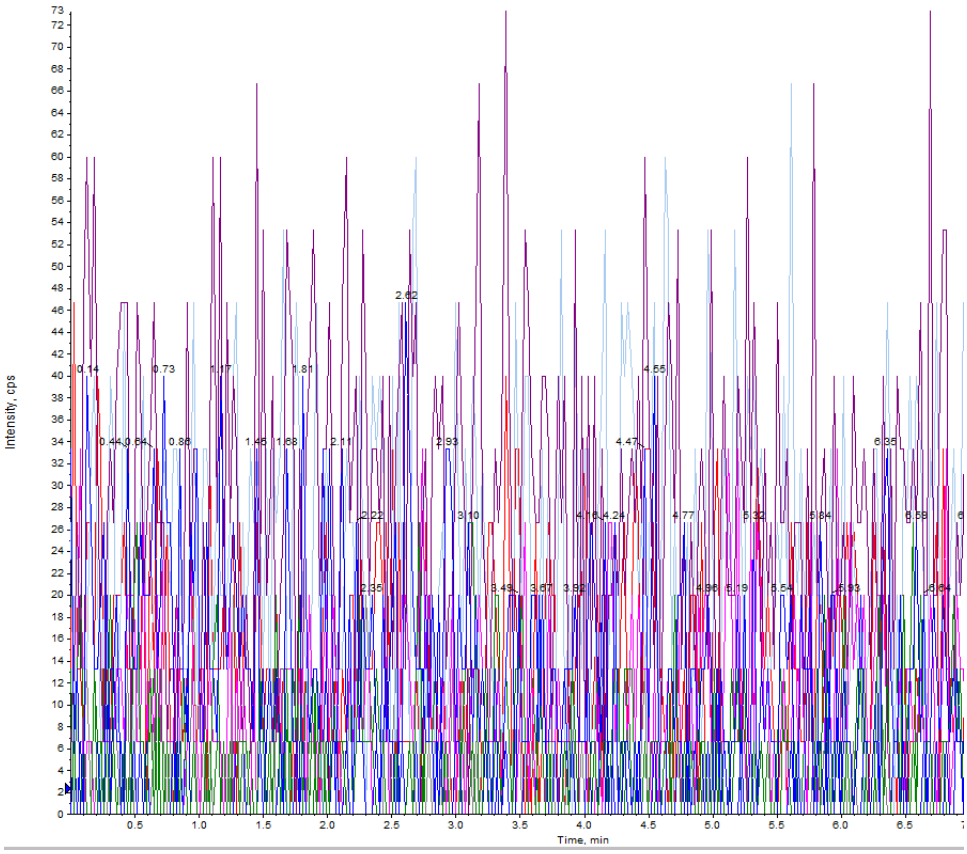

Figure 27: Example chromatogram of the absence of peaks after injections of 0.50 mg/L, demonstrating the lack of carryover

3.5.8. Autosampler stability

The results from this experiment were plotted on graphs to evaluate autosampler stability. Figures 28 and 29 shows all analytes for the low (0.015 mg/L) and the high (0.15 mg/L) concentration, respectively. The response did not fall below 20% of the time zero response (t0) within 48 hours in a temperature controlled room (16°C - 24°C) and all analytes were considered stable under these conditions.

Lormetazepam appeared to be the least stable and showed a downward trend for both concentrations compared to the other analytes however the responses for all time points were within 20% of t0.

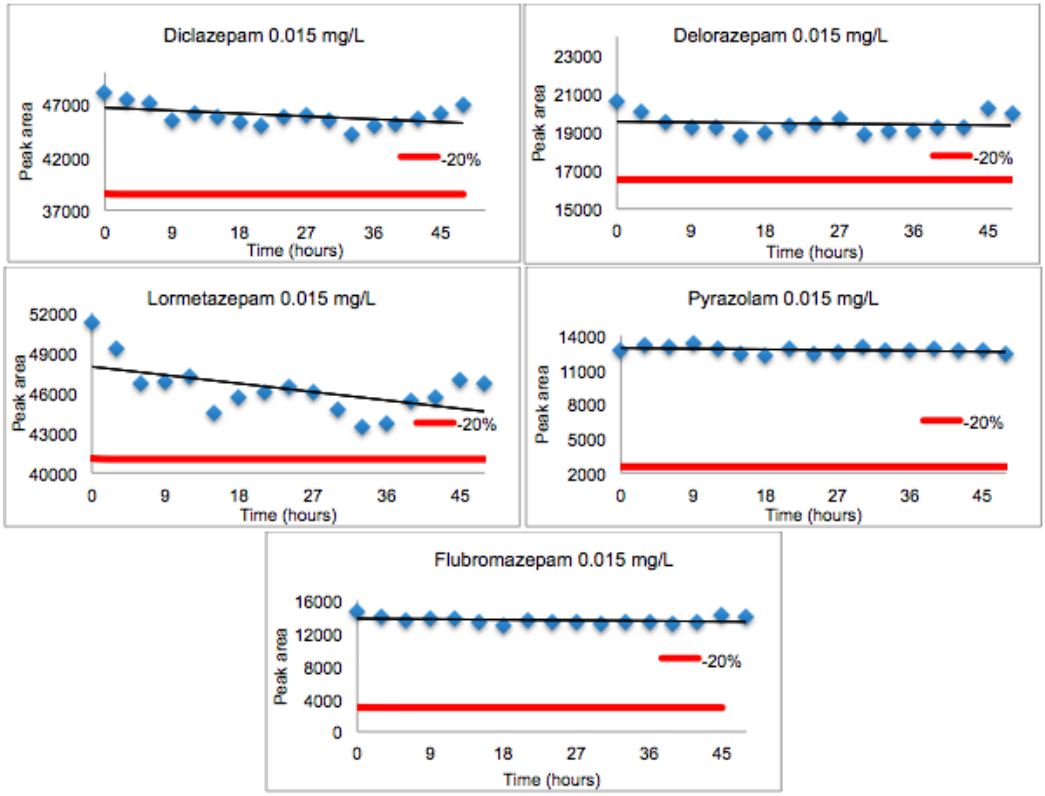

Figure 28: Autosampler stability for all blood method analytes at a concentration of 0.015 mg/L over 48 hours at 16-24 °C

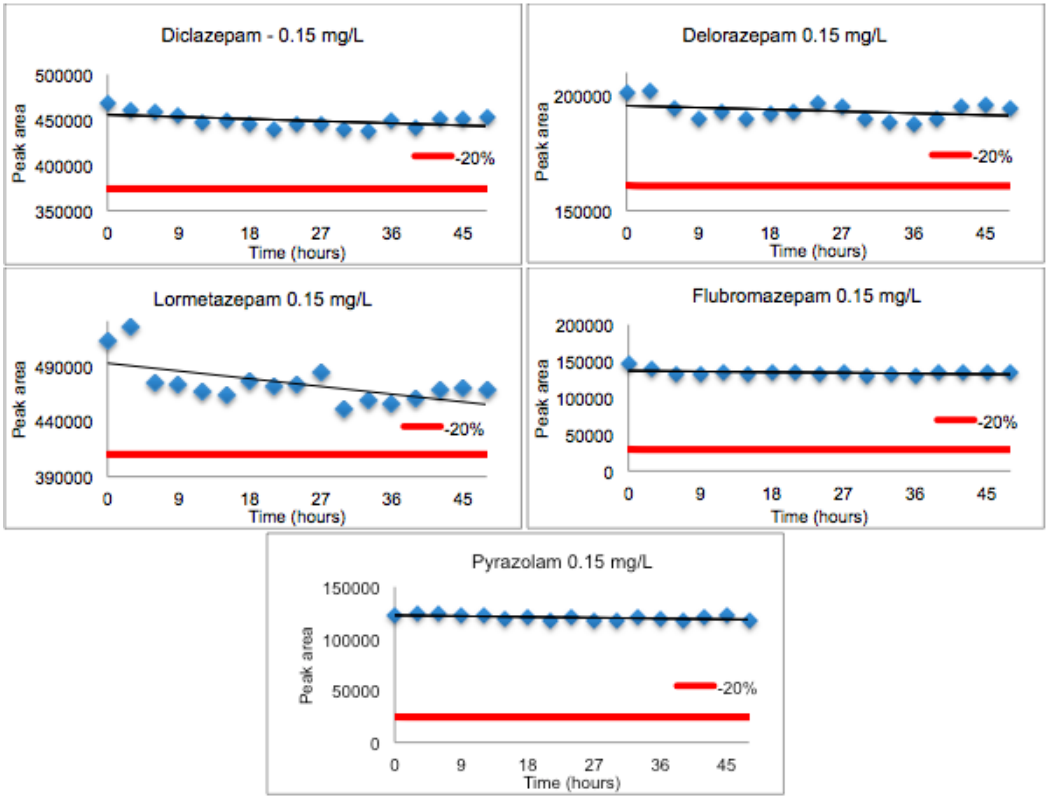

Figure 29: Autosampler stability for all blood method analytes at a concentration of 0.15 mg/L over 48 hours at 16-24 °C.

3.6. Conclusion

This method is suitable for the quantitative analysis of blood samples for diclazepam and its metabolites (delorazepam and lormetazepam), flubromazepam and pyrazolam. The method spans a concentration range (0.005-0.20 mg/L) for these drugs in blood with acceptable linearity. The method is specific and selective, and demonstrated no carryover at the concentration tested. Bias and precision were acceptable for all analytes. Matrix effects were found to be acceptable for all analytes and within the ±25% criterion for %ME and <15% for precision. Recovery and process efficiency was suitable for diclazepam and delorazepam, it was suboptimal for lormetazepam and flubromazepam however the bias, precision and sensitivity were good. Pyrazolams recovery and process efficiency was particularly low and this should be considered when analysing real samples in particular decomposed samples as they may give a lower concentration and samples which are positive at the lower end of the concentration range may give a negative result. All analytes were stable in the autosampler stability up to 48 hours in a laboratory with the temperature range 16-24 °C. A temperature-controlled autosampler would allow more control over the analyte stabilty however it has been demonstrated that analysis within 48 hours should not be an issue. As further work, freeze and thaw stability should be investigated for these analytes, as the long-term stabilty in blood is still unknown.

4. Development and validation of a qualitative LC-MS/MS method for the detection of designer benzodiazepines in urine

4.1. Introduction

The first step in method development is to establish the purpose of the method, as this should dictate the subsequent decisions such as what sample preparation, chromatographic and detection system to use. It is important to choose relevant drugs/metabolites, internal standards and an appropriate sample matrix. In order to detect designer benzodiazepines in urine it was important that a robust method was developed and validated in order to acquire the qualitative results for the projects detailed in chapters 5 and 6 of this thesis.

4.2. Aim

The aim of this work was to develop and validate a qualitative LLE, LC-MS/MS method for the detection of benzodiazepines including designer benzodiazepines in urine.

4.3. Materials and Methods

4.3.1. Materials

Diclazepam (1mg/mL in MeOH, >99%), flubromazepam (1mg/mL in MeOH, >99%), pyrazolam (1mg/mL in ACN, >99%), nifoxipam (1mg/mL in MeOH, >98%), clonazolam (1mg/mL in MeOH, >99%), flubromazolam (1mg/mL in MeOH, >99%), deschloroetizolam (1mg/mL in MeOH, >99%), meclonazepam (1mg/mL in MeOH, >99%), 3-hydroxyphenazepam (1mg/mL in MeOH, >99%) and metizolam (0.1mg/mL in MeOH, >99%) were purchased from Chiron (Trondheim, Norway). Chlordiazepoxide (1mg/mL in MeOH, >99%), nitrazepam, (1mg/mL in ACN, >99%) alprazolam (1mg/mL in MeOH, >99%), lorazepam (1mg/mL in ACN, >99%), oxazepam, (1mg/mL in MeOH, >99%) temazepam (1mg/mL in MeOH, >99%), lormetazepam, (1mg/mL in MeOH, >99%), delorazepam (0.1mg/mL in MeOH, >99%), etizolam (1mg/mL in MeOH, >99%), phenazepam (1mg/mL in ACN, >99%), desmethyldiazepam (1mg/mL in MeOH, >99%) and diazepam (1mg/mL in MeOH, >99%) were purchased from Sigma Aldrich (Gillingham, UK). Deionised water was prepared in-house using the Purite (Thame, UK) deionised water system. Disodium hydrogen orthophosphate anhydrous (AR Grade) and sodium dihydrogen orthophosphate dihydrate (AR grade), sodium acetate trihydrate (AR grade) and glacial acetate acid (AR grade, >99.7%) were purchased from Fisher

Scientific (Loughborough, UK). Tertiary methyl butyl ether (tBME) (anhydrous grade, >99%), β-glucuronidase from *Helix Pomatia* (≥100,000 units/mL) and ammonium acetate (AR grade) were purchased from Sigma Aldrich (Gillingham, UK). Methanol (HPLC grade), acetonitrile (LC/MS grade), formic acid (AR grade, >99%) and ammonia (AR grade, 28%) were purchased from VWR (Lutterworth, Leicestershire, UK). Blank (drug-free) urine was provided anonymously by in-house donors. The blank urine collection process was approved by the University of Glasgow Medical, Veterinary and Life Sciences ethics committee under reference No. 200160020. (See appendix 1.1).

4.3.2. Reagent preparation

Table 33 details how the reagents used for the urine method were prepared.

Table 33: Urine method reagent preparation

Reagents	Substance 1	Substance 2	Substance 3	Substance 4	Storage
Sodium acetate buffer (pH 5.0, 0.1M)	Sodium acetate trihydrate *5.86g*	DiH_2O *Up to 1000 mL*	Glacial acetic acid (for pH adjustment)	-	Room Temp.
Phosphate buffer (pH 6, 0.1M)	Disodium hydrogen orthophosphate anhydrous *1.7 g*	Sodium dihydrogen orthophosphate dihydrate *12.14 g*	DiH_2O *Up to 1000 mL*	0.1M monobasic/dibasic sodium phosphate (for pH adjustment)	Room Temp.
0.1M monobasic sodium phosphate	Sodium dihydrogen orthophosphate dihydrate 2.76 g	DiH_2O *Up to 150 mL*	-	-	Fridge (2-8°C)
0.1M dibasic sodium phosphate	Disodium hydrogen orthophosphate anhydrous 2.84 g	DiH_2O *Up to 150 mL*	-	-	Fridge (2-8°C)
Reconstitution solution (25:75, v:v)	Methanol *50 mL*	DiH_2O *150 mL*	2M ammonium acetate *200 µL*	Formic acid *200 µL*	Room Temp.
2M ammonium acetate	Ammonium acetate *15.4 g*	DiH_2O *100 mL*	-	-	Room Temp.
Infusion solution	Methanol 500 mL	DiH_2O *500 mL*	Formic acid 1 mL	2M ammonium acetate 1 mL	Room Temp.
Mobile Phase A	DiH_2O *1000 mL*	2M ammonium acetate *1 ml*	Formic acid *1 mL*		Room Temp.
Mobile Phase B	Methanol *1000 mL*	2 M ammonium acetate *1 ml*	Formic acid *1 mL*		Room Temp.

All solutions were inverted to mix then transferred to appropriate containers.

4.3.3. Drug and internal standard preparation

Table 34 shows how the benzodiazepine drug stock solutions were prepared. Table 35 details how working drug solutions (high and low QCs, LOD and autosampler stability) were prepared using the benzodiazepine drug stock solution.

Table 34: Urine method benzodiazepine drug stock solution preparation

	Drug @ 1 mg/mL ***100 µL added***	**Drug @ 100 µg/mL** ***1 mL added***	**Solvent** ***Up to 10 mL***	**Storage**
Stock Solution @ 10 µg/mL	Pyrazolam Nifoxipam Chlordiazepoxide Clonazolam Nitrazepam Alprazolam Flubromazolam Lorazepam Deschloroetizolam Etizolam Oxazepam Meclonazepam Temazepam Lormetazepam Flubromazepam Desmethyldiazepam Phenazepam Diclazepam Diazepam 3-hydroxyphenazepam	Metizolam Delorazepam	Methanol	Freezer (≤-20 °C)

Table 35: Urine method drug working solution preparation

Solution	**Substance 1**	**Substance 2**	**Storage**
Low QC (0.075 µg/mL)	Stock solution (10 µg/mL) *75 µL*	Methanol *Up to 10 mL*	Freezer (≤-20 °C)
High QC (0.40 µg/mL)	Stock solution (10 µg/mL) *0.4 mL*	Methanol *Up to 10 mL*	Freezer (≤-20 °C)
LOD and autosampler stability (1 µg/mL)	Stock solution (10 µg/mL) *1 mL*	Methanol *Up to 10 mL*	Freezer (≤-20 °C)

Tables 36 and 37 detail the preparation of the internal standard stock and working solutions.

Table 36: Urine method internal standard stock preparation

Internal standard stock solution @ 10 µg/mL	Drug @ 100 µg/mL *1 mL added*	Solvent *Up to 10 mL*
	Diazepam-D5 Lorazepam-D4 7-aminoflunitrazepam-D7	Methanol

Table 37: Urine method internal standard working solution preparation

Solution	Substance 1	Substance 2
Internal standard working solution @ 1 µg/mL	Internal standard stock solution @ 10 µg/mL *1 mL*	Methanol *Up to 10 mL*

4.3.4. LC-MS/MS instrumentation and parameters

The analysis was carried out using an Agilent 1200 LC system coupled to an AB Sciex 3200 QTRAP MS. The software utilised was Analyst® 1.7. The column oven was set at 40 °C and was fitted with a Phenomenex Gemini C18 column (150 mm x 2.0 mm, 5 µm) which had a pre-column SecurityGuard™ cartridge (4.0 mm x 2.0 mm) in place. A gradient system consisting of two mobile phases; 2mM ammonium acetate and 0.1% formic acid in diH_2O water (mobile phase A) and 2mM ammonium acetate and 0.1% formic acid in methanol (mobile phase B) was used.

Positive electrospray ionisation (turbo ion spray is the term used on the analyst software) was utilised and the ion source temperature was maintained at 350°C. Nitrogen was used as the source and collision gas. The collision gas (CAD) was set at 4 and the ion source gas 1 and 2 were set at 30 psi. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode.

4.4. Qualitative method experimental

4.4.1. Method development experimental

4.4.1.1. MS/MS optimisation

In order to identify and optimise the ion transitions and instrument settings for all analytes, each drug and internal standard were directly infused into the mass spectrometer at the rate 10 µL/min at a concentration of 1 µg/mL in infusion solution using the instruments inbuilt infusion pump. The instrument operating in triple quadrapole mode was set a range to scan to identify the precursor ion using the molecular weight of the drug, e.g diclazepam molecular weight is 319.18 so the scan range can be set to 270 – 370 Da initially to find the ion, then the range was narrowed as required. The product ion scan is then performed again using a suitable range; all the drugs in this method were scanned for fragments greater than 125 Da. The compound optimisation wizard was then set to determine the optimum settings for the four most abundant fragments within the mass range selected. The optimal product ion can then be selected from these four to form the method with the settings associated with that analyte. (AB Sciex, 2011) This selection is a compromise between sensitivity and specificity; the most abundant ion is not always the most optimal choice. For each MRM transition, the following parameters were optimised: declustering potential (DP), entrance potential (EP), collision entrance potential (CEP), collision energy (CE) and collision exit potential (CXP). The DP is the voltage which is applied at the source orifice to prevent all the ions clustering together. The EP is the next voltage applied after the ions have entered the orifice and helps to focus the ion's pathway. CE is the rate of acceleration in Q2, if the CE is too low fragmentation will be inefficient however extensive fragmentation can occur if it is too high. CEP and CXP are the collision entrance and exit potential respectively; these are responsible for accelerating and focussing the ions into and out of Q2, into to Q3 in the case of CXP. (AB Sciex, 2011)

4.4.1.2. LC gradient

As the method has multiple benzodiazepines, some of which have a very similar structure, an isocratic method was unlikely to give the chromatographic resolution required within an acceptable run-time. Therefore use of a gradient was investigated. The mobile phase compositions and column selection were fixed to

allow it to be integrated to the FMS routine laboratory; therefore these were not further investigated for development.

An unextracted mix of all the analytes plus the internal standards was prepared at a concentration of 0.1 mg/L in reconstitution solution. For each gradient programme tested this mix was injected at least four times to assess if the gradient was been sufficient to elute all analytes. A total of 22 gradients and 2 isocratic programmes were investigated. The two isocratic programmes were at 60 and 30% mobile phase (MP) B. Of the 22 gradients, 18 were started at 30 or 40% MP B and contained ramps ranging from 40 to 90% MP B with run times ranging from 36 to 55 minutes, these are further described in section 4.5.1.2. The other four gradients were started at 10 or 20% MP B and contained ramps ranging from 40 to 90% MP B with run times ranging from 40 to 60 minutes. The aim was to use a gradient system with good chromatographic resolution with suitable time between some peaks so time periods could be set up. Altering the MRM dwell time from 125 msecs to 100 msecs was also investigated.

4.4.1.3. Sample extraction

The extraction for this method needed to be fast, simple and cost effective as a large number of samples had to be analysed using this method (all samples received from the SPS, SDC and NHS GGC FD, see chapters 5 and 6) therefore an in-house LLE procedure was utilised. The volume of extraction solvent *t*BME was assessed but otherwise there was no further extraction development. In order to assess the volume of solvent a %RE and %ME experiment was carried out. The equations for %RE and %ME are given in section 3.4.6 in chapter 3. The two volumes that were investigated were 0.75 mL and 1.75 mL of *t*BME. The volume 1.75 mL was selected as this was just below 2 mL but allows some room in the 4 mL tubes used for efficient mixing without overflowing. The lower volume 0.75 mL was then selected, as it is a 1 mL difference.

- Set A: neat unextracted with low QC and high QC separately and internal standard in reconstitution solution was prepared in duplicate.
- Set B: blank (drug free) urine was spiked with low QC and high QC separately and internal standard in reconstitution solution after LLE. Set B was prepared in duplicate for each of the two volumes of *t*BME being evaluated.
- Set C: blank (drug free) urine was spiked with low QC and high QC separately and internal standard prior to LLE. Set C was prepared in duplicate for each of the two volumes of *t*BME being evaluated.

4.4.1.4. Hydrolysis step optimisation

The use of beta-glucuronidase is well established in benzodiazepine urinalysis (De Jager and Bailey, 2011; Marin and McMillin, 2010; Bergstrand, Helander and Beck, 2016), the object of this experiment is determine if a lower volume of beta-glucuronidase can be used for this study compared to the usual FMS procedure. The current FMS benzodiazepine urine extraction uses 40 µL of beta-glucuronidase from *Helix Pometia* but also utilises 1 mL of urine sample and is extracted using SPE. As the method developed for this study would use 0.5 mL of urine sample and use a LLE, a reduced volume of beta-glucuronidase would be advantageous; it would be less expensive and give a cleaner extract. In order to test if the volume of beta-glucuronidase used could be decreased, three real positive urine samples were extracted, once using 20 µL beta-glucuronidase in duplicate and once using 40 µL beta-glucuronidase in duplicate giving a total of 12 sample tubes. The real urine samples were from the SDC cohort; results of the drug court study are detailed in chapter 5. The samples were chosen as the individual had admitted to taking diazepam on their questionnaire so were anticipated to give positive results. Figure 30 shows the process used to carry out this experiment.

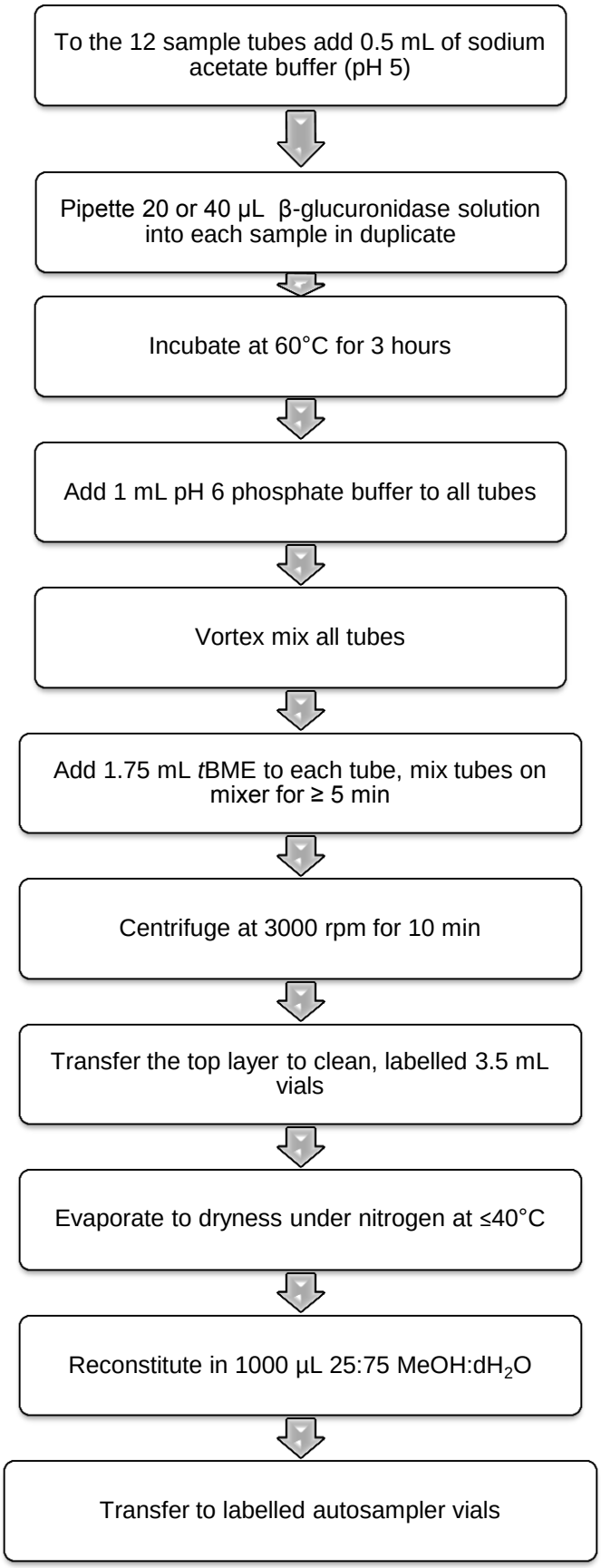

Figure 30: Hydrolysis step optimisation LLE process

4.4.2. Method validation experimental

The validation parameters were evaluated for the final LLE method detailed in section 4.5.2 and the final LC-MS/MS method detailed in section 4.3.4. The parameters evaluated were limit of detection, specificity, matrix effects, recovery and process efficiency, carryover and autosampler stability.

4.4.2.1. Limit of detection

The LOD study was carried out in the same way as described in section 3.4.4 of chapter 3. LOD for each analyte was evaluated. Extracted concentrations of 0.002, 0.004, 0.006 and 0.008 mg/L were tested in duplicate. The signal-to-noise ratio should be ≥3 for the analyte to be considered detectable. Retention time for each analyte should be consistent.

4.4.2.2. Specificity

In order to understand if the benzodiazepine drugs would create interference issues within the detection window of each drug within the urine method, a selection of benzodiazepines including all drugs contained in the method plus flunitrazepam, flurazepam, clozapine, midazolam, clobazam, prazepam, 7-aminoflunitrazepam and clonazepam were individually injected, as an unextracted 0.10 mg/L solution. Each drug was injected in triplicate.

4.4.2.3. Matrix effects, recovery and process efficiency

Matrix effects, recovery and process efficiency are described in section 3.4.6 of chapter 3.

For two concentrations investigated, ME, RE and PE were calculated using the post-extraction addition approach detailed by Matuszewski *et al.* (Matuszewski, Constanzer and Chavez-Eng, 2003)

Blank (drug free) urine from 10 different sources was used for the experiments. The analytes at two different concentrations (ME Low QC 0.015 and ME High QC 0.40 mg/L) were evaluated. The ME, RE and PE experiments were performed as follows:

- Set A: neat unextracted analyte mix and internal standard in reconstitution solution was prepared ten times.
- Set B: blank urine from 10 different sources were spiked with the analyte mix and internal standard in reconstitution solution after LLE. Set B was prepared in duplicate.
- Set C: blank urine from 10 different sources were spiked with analyte mix and internal standard prior to LLE. Set C was prepared in duplicate.

4.4.2.4. Carryover

Carryover is described in section 3.4.7 of chapter 3.

A solution was prepared to give a concentration of 4 mg/L for all 22 analytes, this was extracted using the LLE method and injected in triplicate followed, by three injections of drug-free reconstitution solution. The concentration 4 mg/L was chosen, as this is ten times the high QC concentration and double the concentration of benzodiazepines used in the FMS in-house method.

4.4.2.5. Autosampler stability

Blank (drug free) urine was spiked using the AS stability solution at a high (0.150 mg/L) and the low (0.015 mg/L) concentration in triplicate and extracted using the LLE procedure detailed in section 4.5.1.3 These extracts were then run on the method detailed in section 4.3.4 to determine a time zero response (t0); the same extracts were then injected at four-hour intervals up to 36 hours. The laboratory was temperature controlled between 16°C and 24°C during the experiment.

4.4.3. Data handling and statistical analysis

Once results had been generated in the Analyst software, they were copied onto a Microsoft® Excel® (version 14.7.3) spreadsheet and batch checked by a second toxicologist before any calculations were performed. The results copied include the peak areas of the analyte and the internal standard, the calculated values of the calibration standards and QCs in mg/L and the datapath information. Microsoft® Excel® was then used to calculate the mean, standard deviation, %CV, %ME, %PE and %RE where necessary. A second toxicologist, as part of the batch checking, also checked the formula used in the spreadsheets for each calculation. Line graphs were generated in the spreadsheet to track autosampler stability. The Statsplus (AnalystSoft™, version 7.7.31) add on for Microsoft® Excel® was used

to conduct a paired two sample t-test to determine if there was a statistical difference between the volumes of *t*BME used. The spreadsheets were saved on a secure drive to protect the data.

4.5. Results and discussion

4.5.1. Method development

4.5.1.1. MS/MS Optimisation

The identified MRM transitions and optimised MS parameters for all analytes in the method are displayed in Table 38.

The method contains 22 benzodiazepine drugs/metabolites with 2 MRM transitions for each as well as 3 internal standards for which one MRM was monitored. The MS parameters in Table 38 gave the optimal response for all analyte MRMs and the precursor ion identified for all analytes was $[M+H]^+$. Two product ions, which gave appropriate sensitivity and specificity, were selected to form the quantifier (QT) and qualifier (QL) MRMs. The MS resolution used in Q1 for all analytes was unit and low for Q3.

Table 38: Urine method MRM transitions and optimised MS parameters

Analyte	Q1	Q3	DP	EP	CEP	CE	CXP
Pyrazolam-QT	354.2	206.2	61	11.0	16	43	6
Pyrazolam-QL	354.1	167.0	61	11.0	16	45	6
Nifoxipam-QT	316.1	270.2	51	5.0	16	25	4
Nifoxipam-QL	316.1	298.2	51	5.0	16	21	4
Chlordiazepoxide-QT	300.1	227.2	41	8.0	14	35	4
Chlordiazepoxide-QL	300.1	241.2	41	8.0	14	23	6
Clonazolam-QT	354.0	308.2	61	5	30	27	6
Clonazolam-QL	354.0	280.1	61	5	30	47	4
Nitrazepam-QT	282.1	236.3	66	10.5	14	35	4
Nitrazepam-QL	282.1	180.3	66	10.5	14	51	4
Alprazolam-QT	309.1	281.3	61	10.5	14	37	6
Alprazolam-QL	309.1	205.2	61	10.5	14	55	6
Flubromazolam-QT	370.9	223.1	81	5.0	26	55	4
Flubromazolam-QL	370.9	343.1	81	5.0	26	55	6
Lorazepam-QT	321.0	229.2	46	10.5	14	31	4
Lorazepam-QL	321.0	275.2	46	10.5	14	39	4
Deschloroetizolam-QT	309.2	280.1	66	8.5	14	33	4
Deschloroetizolam-QL	309.2	255.2	66	8.5	14	33	4
Etizolam-QT	343.1	314.3	66	10.5	14	33	6
Etizolam-QL	343.1	259.1	66	10.5	14	45	6
Oxazepam-QT	287.0	241.2	46	9.0	12	33	4
Oxazepam-QL	287.0	269.2	46	9.0	12	19	4
Meclonazepam-QT	330.1	284.1	66	8	22	35	4
Meclonazepam-QL	330.1	214.2	66	8	22	51	4
Temazepam-QT	301.1	255.1	46	8.0	14	33	4
Temazepam-QL	301.1	283.2	46	8.0	14	17	4
Lormetazepam-QT	335.1	289.1	46	10.5	16	31	6
Lormetazepam-QL	335.1	177.0	46	10.5	16	55	6
Flubromazepam-QT	333.0	226.2	71	10.5	14	43	6
Flubromazepam-QL	333.0	184.0	71	10.5	14	41	6
Delorazepam-QT	305.1	140.0	66	10.5	12	41	6
Delorazepam-QL	305.1	206.1	66	10.5	12	49	6
DMD-QT	271.1	140.0	66	10.0	12	39	4
DMD-QL	271.1	165.1	66	10.0	12	39	4
Phenazepam-QT	348.9	206.2	71	10.5	14	49	6
Phenazepam-QL	348.9	184.1	71	10.5	14	43	6
Diclazepam-QT	319.1	227.2	71	10.5	16	39	6
Diclazepam-QL	319.1	154.1	71	10.5	16	43	6
Diazepam-QT	285.1	193.1	61	10.5	14	43	4
Diazepam-QL	285.1	154.1	61	10.5	14	37	4
3-hydroxyphenazepam-QT	364.9	319.1	51	5	22	27	6
3-hydroxyphenazepam-QL	364.9	273.0	51	5	22	43	6
Metizolam-QT	329.0	275.3	66	8	18	41	4
Metizolam-QL	329.0	300.0	66	8	18	41	6
Lorazepam-D4	325.1	279.2	51	10.0	14	33	6
Diazepam-D5	290.1	198.1	66	10.5	14	45	4
7-aminoflunitrazepam-D7	291.2	138.3	56	11.0	12	39	4

Benzodiazepines have naturally occurring isotopes present within their structure, which can be observed when using MS. Two of which are chlorine present as ^{35}Cl and ^{37}Cl and bromine present as ^{79}Br and ^{81}Br. Since ^{35}Cl is 76% abundant versus ^{37}Cl 24% abundance the ^{35}Cl precursor ion was used in the final method. Bromine isotopes are almost even in abundance with ^{79}Br making up 51%. Br^{79} was used in the final method. Table 39 displays if chlorine or bromine is present in the structure of the designer benzodiazepines contained within this urine method.

Table 39: Urine method – chlorine and bromine in the designer benzodiazepines

Analyte	Cl	Br
Pyrazolam	No	Yes
Nifoxipam	No	No
Clonazolam	Yes	No
Flubromazolam	No	Yes
Deschloroetizolam	No	No
Etizolam	Yes	No
Meclonazepam	Yes	No
Flubromazepam	No	Yes
Phenazepam	Yes	Yes
Diclazepam	Yes	No
Delorazepam	Yes	No
3-hydroxyphenazepam	Yes	Yes
Metizolam	Yes	No

Figure 31 shows the initial product ion MS spectra for pyrazolam (MW 354.2) for both the ^{79}Br (354.2) and ^{81}Br (356.2). Figure 32 shows the final product ion MS spectra for both bromine isotopes for pyrazolam, the *m/z* 285 for the ^{79}Br and the *m/z* 287 for the ^{81}Br shows the bromine is still retained on the fragment at this point but are no longer retained for the product ions used in the final method as both *m/z* 206 and *m/z* 167 are present in both the ^{79}Br and ^{81}Br final product ion spectra.

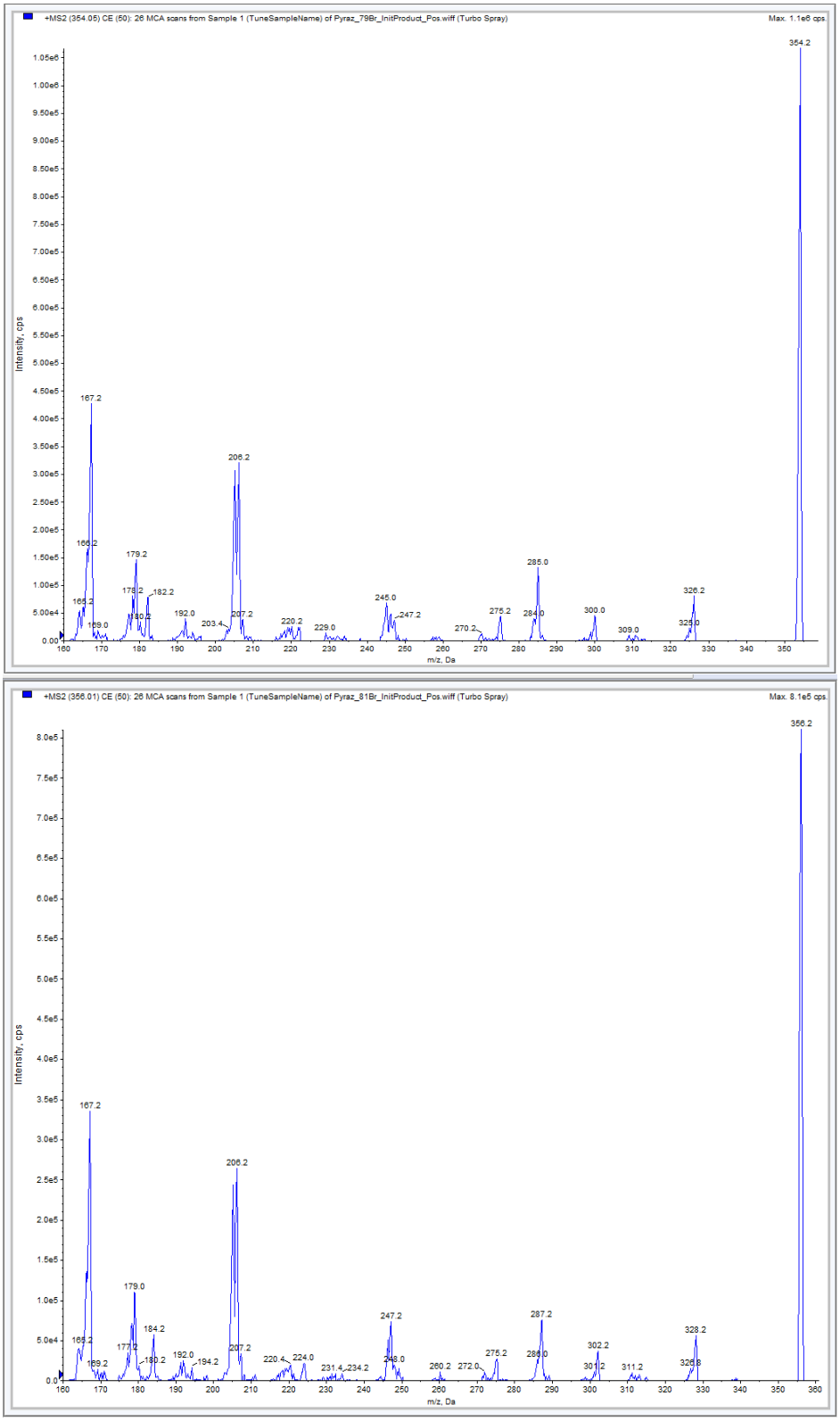

Figure 31: Initial product ion spectra of pyrazolam for both bromine isotopes

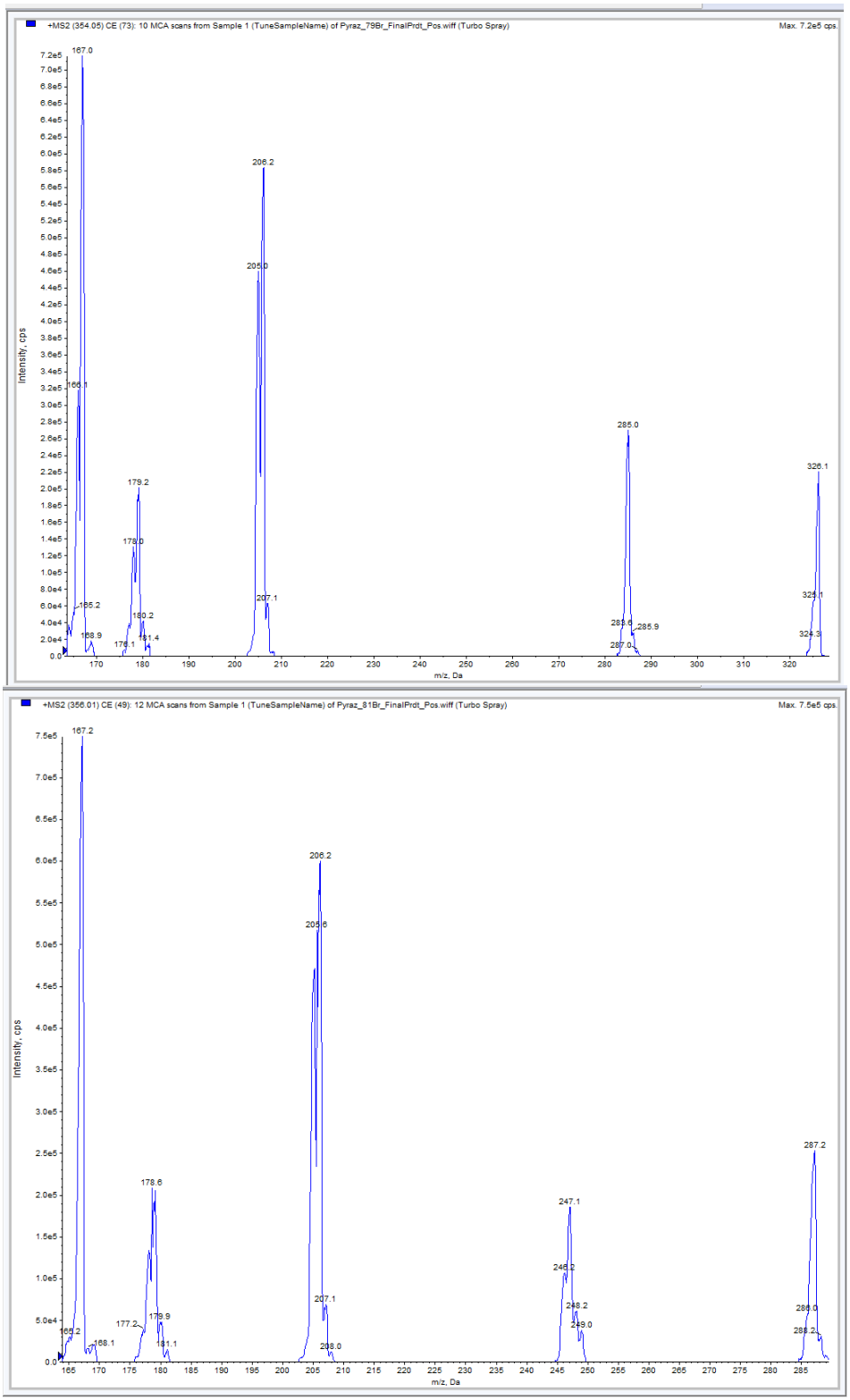

Figure 32: Final product ion spectra of pyrazolam for both bromine isotopes

The fragmentation pathway of each analyte was considered to reinforce the correct selection of transitions and understanding of how the analyte behaved when subjected to the mass spectrometer. Figure 33 shows an example of diclazepam fragmentation. Figure 34 shows the initial and final product ion spectra

for ^{35}Cl diclazepam, the ions m/z 227 and 154 can be seen on the final product scan spectra.

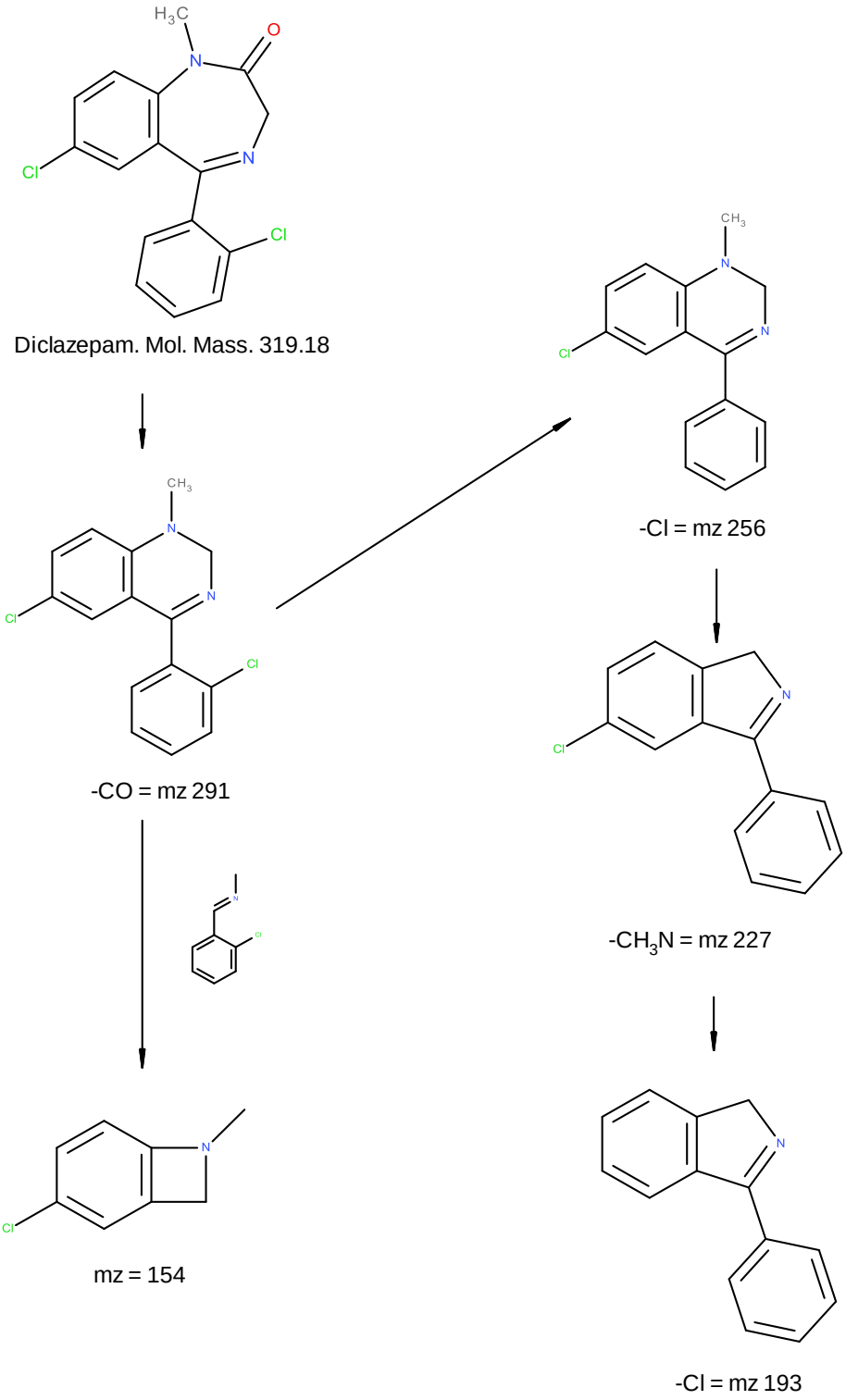

Figure 33: Diclazepam fragmentation pathway

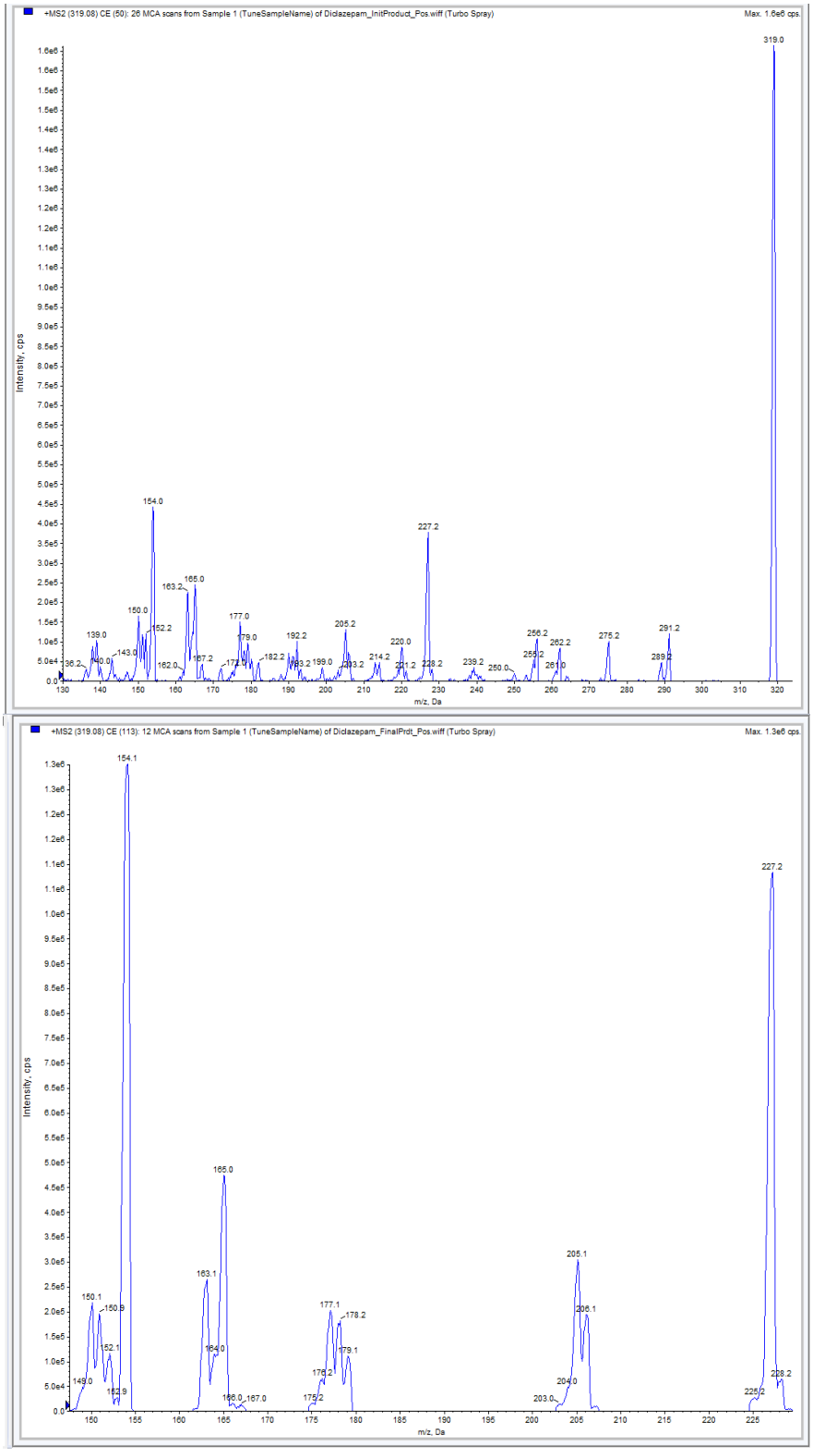

Figure 34: Diclazepam initial and final product ion spectra

4.5.1.2. LC Gradients

The LC was run isocratic at 60% MP B however as expected the analytes co-eluted from the column fairly fast in less than 6 minutes. While this fast run time would have been an advantage it was necessary to slow co-elution down in order to gain better chromatographic resolution. The LC was also run isocratic at 30% MP B and as expected the analytes co-eluted from the column far too slowly with most analytes not eluting at all after 40 minutes, this percentage of organic solvent in an isocratic system is too low for the benzodiazepines. Figure 35 shows an example chromatogram of an injection at 30% MP B.

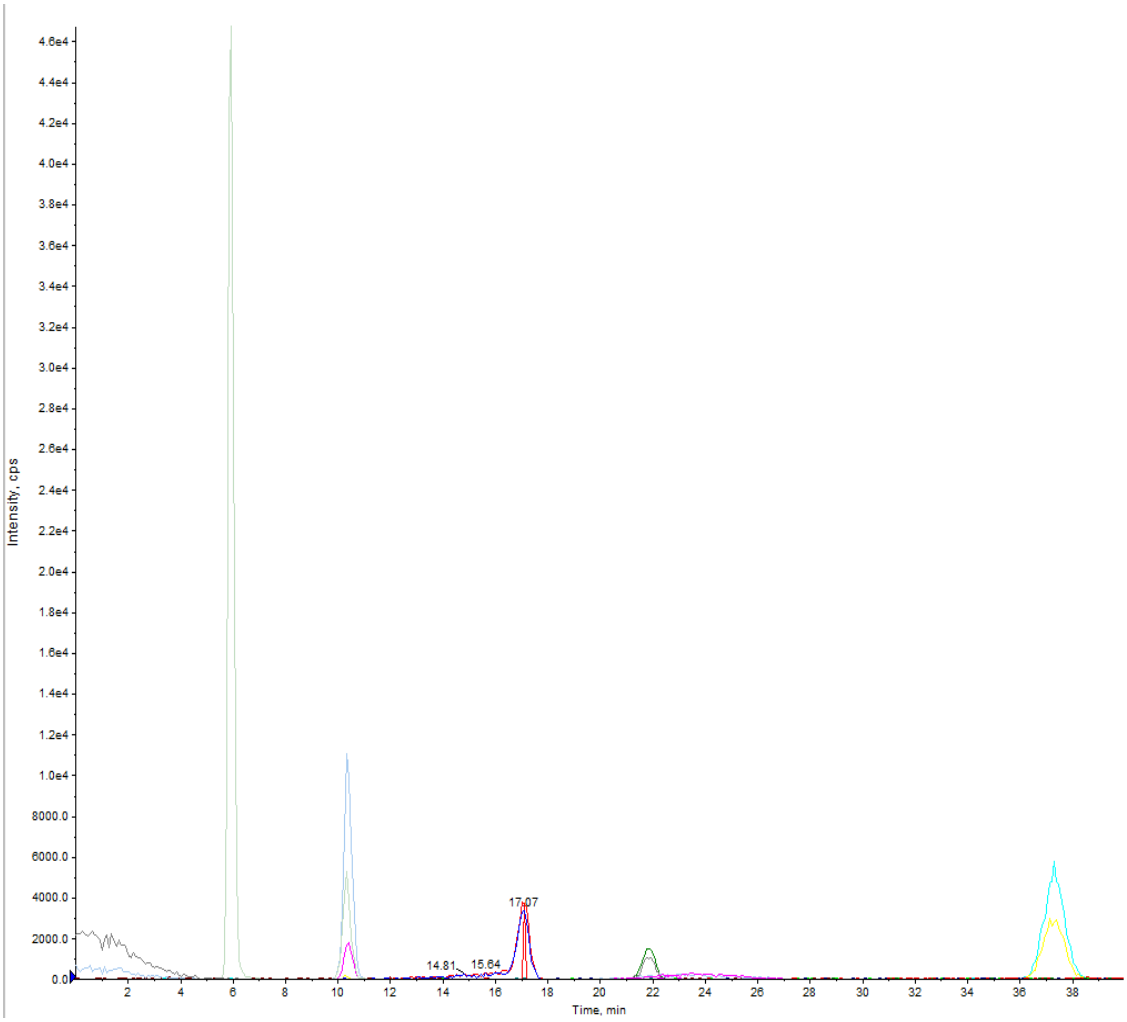

Figure 35: Example chromatogram of a run at 30% mobile phase B.

In total 22 gradients were tested in a variety of combinations, 16 of the combinations are shown in Table 40 with comments on how the analytes behaved, these combinations were all discarded but gives the chronological order to the final gradient decision. The aim was to achieve good chromatographic resolution but

also two gaps in which no peaks eluted in order to divide the analytes in to MS time periods. Analytes clustered together may also increase ion suppression which should be avoided. The different combinations were investigated in order to ascertain the best gradient to meet this goal. Four other gradients tested were started at 10 or 20% MP B and contained ramps ranging from 40 to 95% MP B with run times ranging from 40 to 60 minutes. Figure 36 displays an example chromatogram of a gradient programme starting at 10% MP B. Starting at low percent solvents such as 10 or 20% had no advantage for a benzodiazepine method as the analyte will retain on the column and then will all co-elute quickly once more solvent is introduced.

Table 40: Urine method –LC gradient programme combinations investigated.

Gradient programme	% MP B at start	Description of % MP B	Total run time (mins)	Comments
1	40	Ramped from 40 to 60 steadily over 30 mins - dropped to 40 over 5 mins	40	Fairly good chromatographic separation, a lot of co-elution at 16 mins
2	40	Held at 40 for 14 mins - ramped to 90 steadily over 16 mins - dropped to 40 over 10 mins	40	Most analytes co-elute at 20 to 26 mins
3	30	Held at 30 for 10 mins - ramped to 70 steadily over 12 mins - held at 70 for 6 mins- dropped to 30 over 10 mins	40	Most analytes co-elute at 24 to 28 mins
4	30	Held at 30 for 10 mins - ramped to 60 steadily over 18 mins - dropped to 30 over 10 mins	40	Most analytes co-elute at 26 to 32 mins
5	30	Held at 30 for 10 mins - ramped to 40 steadily over 20 mins - dropped to 30 over 10 mins	40	Only some analytes eluted, not enough organic solvent
6	30	Ramped from 30 to 60 steadily over 45 mins -dropped to 30 over 5 mins - held at 30 for 5 mins	55	Long run time, most analytes not eluting until after 20 mins
7	30	Held at 30 for 4 mins - ramped to 40 steadily over 8 mins - dropped to 30 over 28 mins	40	Only some analytes elute, not enough organic solvent
8	30	Held at 30 for 4 mins - ramped to 40 steadily over 8 mins - ramped to 90 over 2 mins- dropped to 40 over 2 mins- dropped to 30 over 11 mins – held at 40 for 13 mins	40	Most analytes co-elute at 17 to 19 mins
9	30	Held at 30 for 4 mins - ramped to 40 steadily over 8 mins - ramped to 90 - held for 8 mins- dropped to 30 - held at 30 for 20 mins	40	Most analytes co-elute at 17 mins
10	30	Ramped from 30 to 45 steadily over 12 mins – held to 45 for 15 mins – dropped to 30-held at 30 for 13 mins	40	Not every analyte eluted, not enough organic solvent.
11	35	Ramped from 35 to 50 steadily over 30 mins - dropped to 35 over 10 mins	40	Peaks from last injection are present at the start of the run, not enough time to equilibrate back to 35% MP B
12	30	Ramped from 30 to 50 steadily over 40 mins - dropped to 30 over 10 mins	50	Good chromatographic resolution, very long method and not every analyte had eluted by the end
13	40	Ramped from 40 to 60 steadily over 10 mins – held at 60 for 2 mins - dropped to 40 over 8 mins - held at 40 for 18 mins	40	Most analytes co-eluted at 12 to 15 mins
14	40	Ramped from 40 to 60 steadily over 10 mins – held at 60 for 2 mins - dropped to 40 over 2 mins-held at 40 for 25 mins	40	Most analytes co-eluted at 12 to 15 mins
15	40	Ramped from 40 to 60 steadily over 5 mins – held at 60 for 2 mins - dropped to 40 over 5 mins-held at 40 for 30 mins	40	An improvement on gradient 13 and 14 in terms of chromatographic resolution as most analytes co-elute at 8 to 15 mins
16	40	Held at 40 for 2 mins - ramped to 50 steadily over 2 mins - ramped to 55 over 11 mins - held for 3 mins- dropped to 40 over 2 mins - held at 40 for 16 mins	36	Chromatographic resolution is better than other gradients tried considering shorter run time, this is a slight variation of the final gradient used.

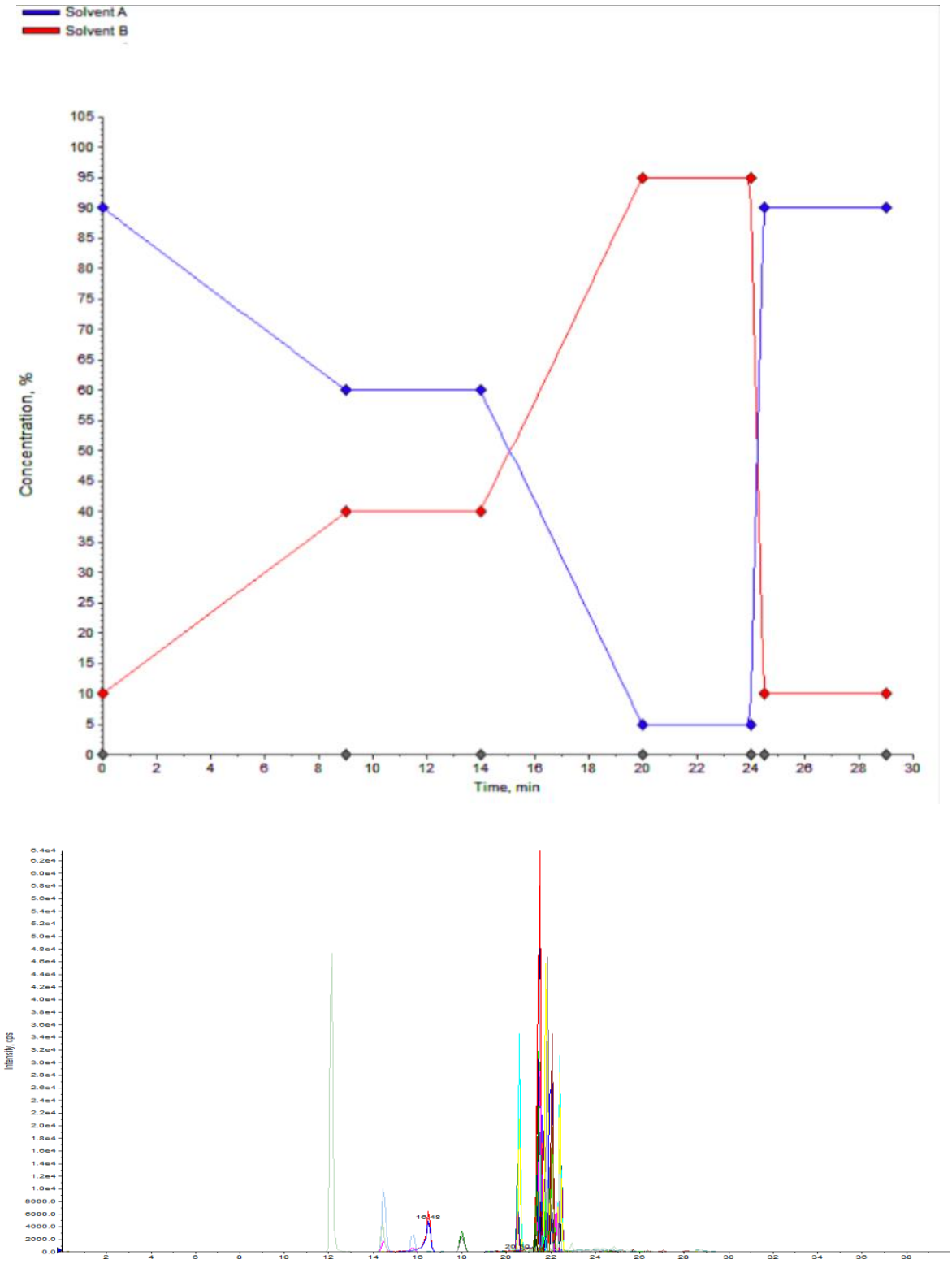

Figure 36: Graphical representation of LC gradient programme starting at 10% MP B and an example chromatogram

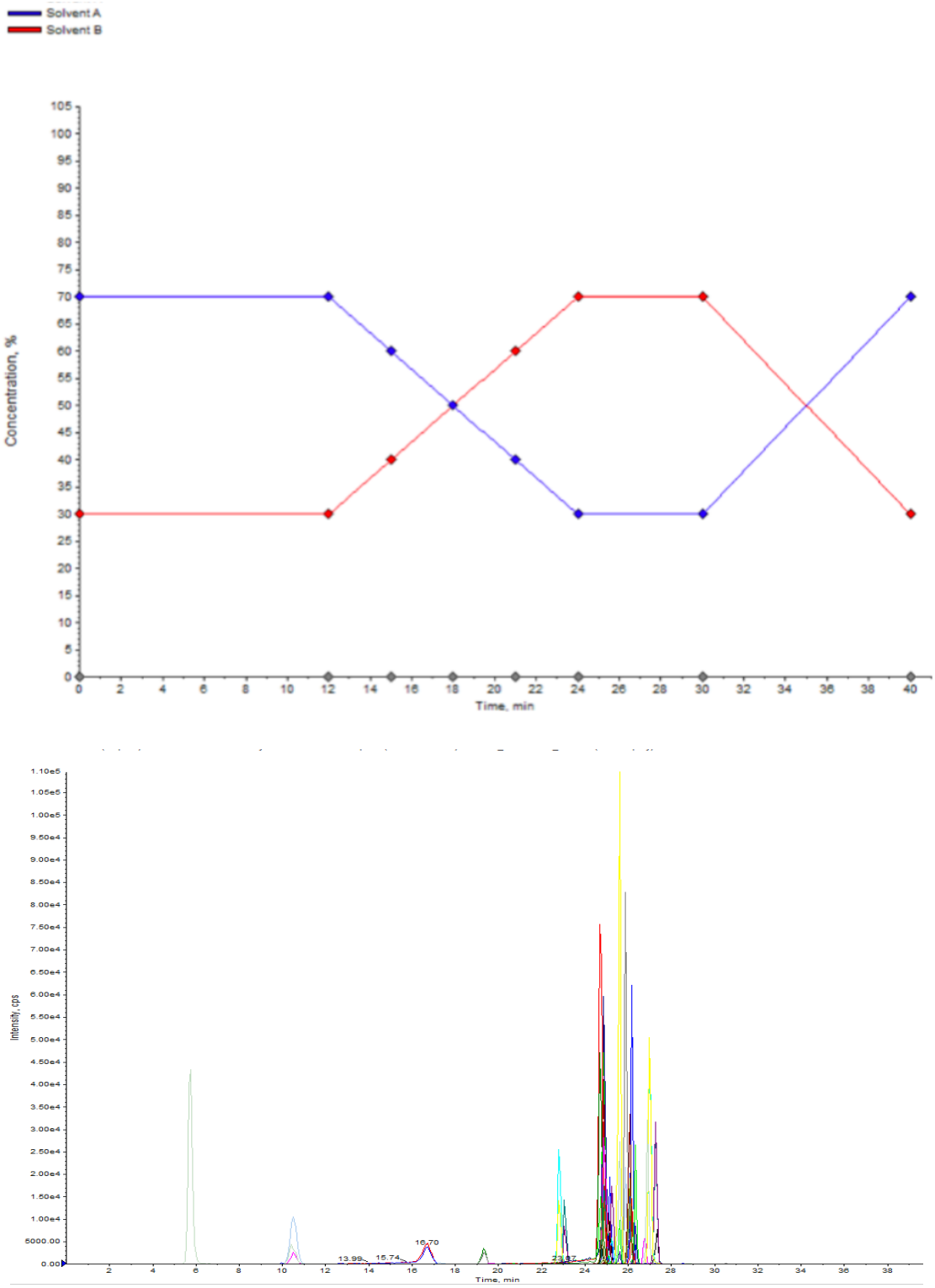

Figure 37: Graphical representation for LC gradient programme 3 and an example chromatogram

Figure 37 shows gradient programme 3 (see Table 40) and an example chromatogram, this gradient is retaining the analytes due to the low organic solvent content at the beginning then they are co-eluting very quickly at the 70%

MP B step of the programme. The analyte peaks have no chromatographic resolution and the run time is not being used efficiently.

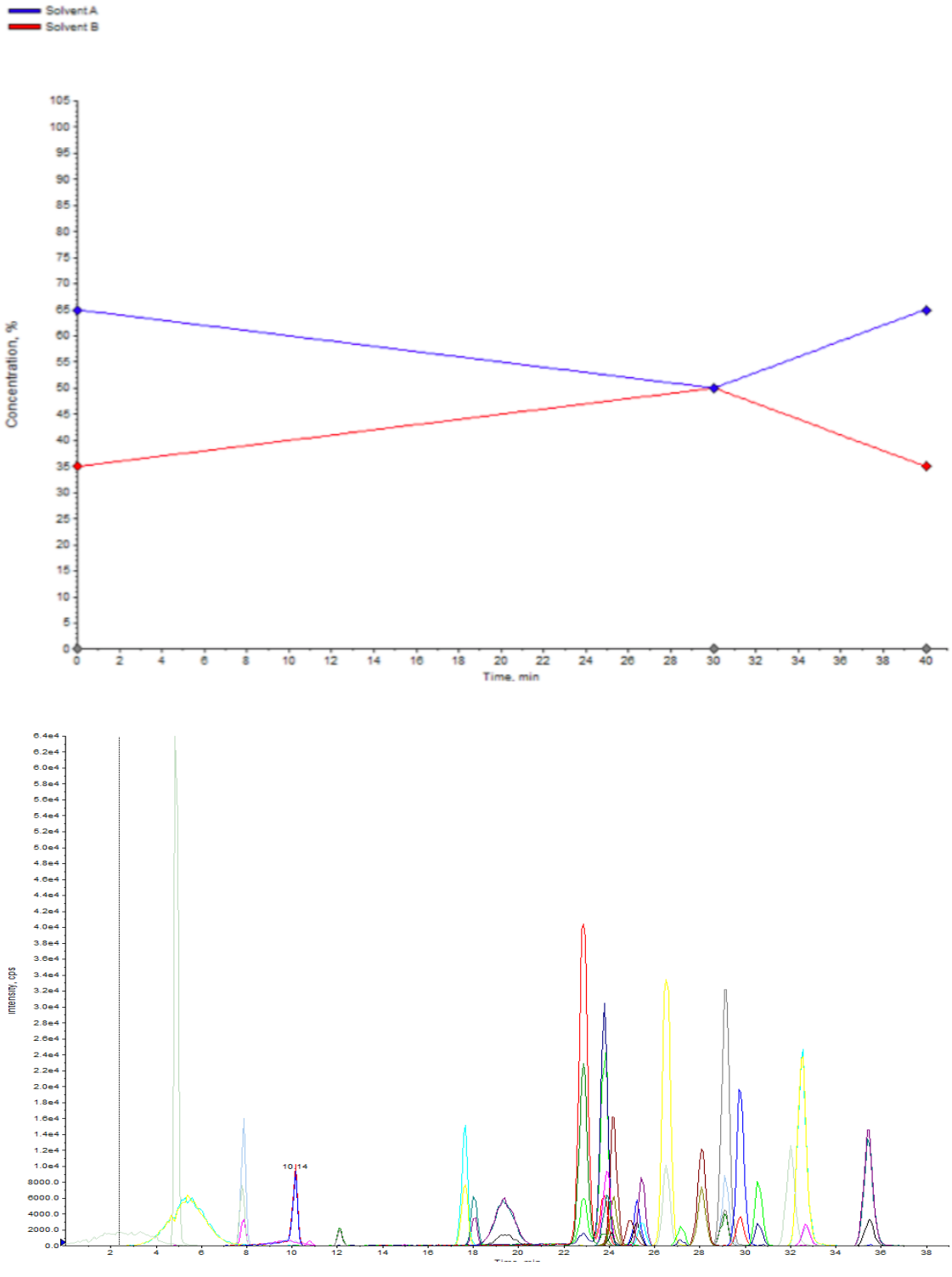

Figure 38: Graphical representation of LC gradient programme 11 and an example chromatogram

Figure 38 shows the gradient programme 11 and an example chromatogram, this gradient gives a good chromatographic resolution of most analytes between 17

mins and 37 mins however the peaks at the start of the chromatograph are from the previous injection. The more retentive analytes at the end of the run are not being eluted in time before the programme switches to decrease the solvent MP B and return to the starting solvent of 35%. This demonstrates the requirement to provide time within the gradient programme for the system to equilibrate back to the starting MP composition.

Gradient 18 was the gradient programme used in the final method and is detailed in Table 41 and Figure 39. This gradient was chosen due to the ability to insert three time periods due to the absence of peaks at particular time points.

The final chosen gradient details are detailed in Table 41. Figure 39 gives a graphical representation and example chromatogram zoomed in show 3 to 23 minutes of the final gradient used in this method.

Table 41: Urine method final LC gradient programme

Time (mins)	Flow rate (µL/min)	A (%)	B (%)
0	300	60	40
0.1	300	60	40
4	300	60	40
8	300	50	50
8.1	300	50	50
22	300	45	55
25	300	45	55
25.1	300	60	40
35	300	60	40

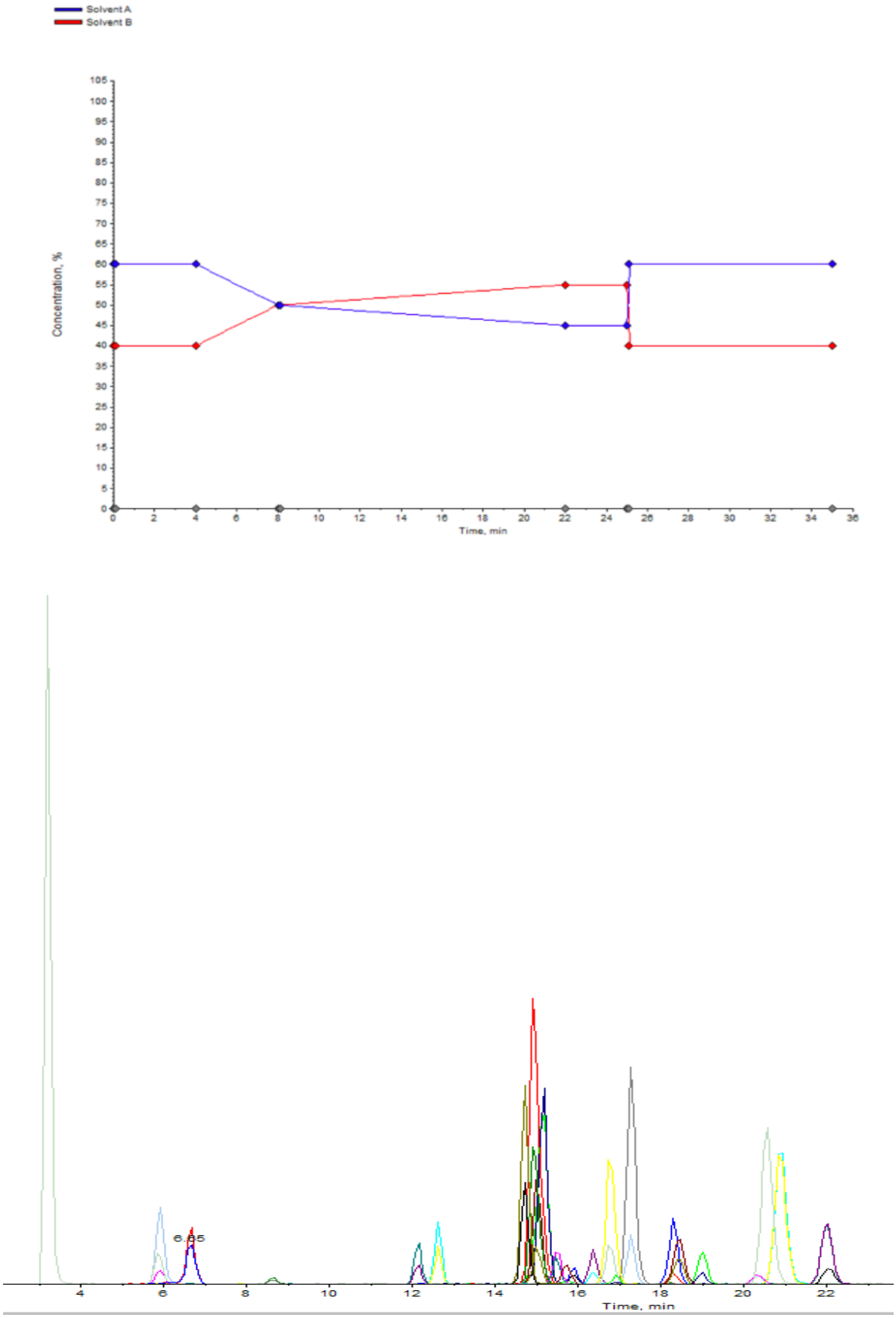

Figure 39: Graphical Representation of the urine method final LC gradient programme and an example chromatogram

Following development of the gradient, the further improvement of creating time periods was made in order to ensure each analyte had a good Gaussian peak shape. As the elution time of each compound was known, the analytes were split up into three defined time periods. Each time period only scans for the expected analytes in that period and therefore disregards the other analytes until it gets to their time period. This allows the instrument to work smarter in multi-analyte methods and improves cycle numbers in each time period. The number of cycles is the number of times it cycles through all the MRMs so the higher number of cycles the better quality of data. As a minimum, 10 data points should be used to generate a peak with 15 to 30 being more preferable and reproducible. Dwell time is the amount of time the instrument is collecting data for each specific MRM. There is a compromise between sensitivity, background instrument noise and enough data points across the peak. Increasing dwell time results in less noise and increased sensitivity but reduces the number of data points across the peak. Decreasing the dwell time results in more points across the peak, as more cycle times can occur as less time is spent on each analyte. A dwell time of 125 msecs was selected for the MRMs in period 1 and 3 as this gave a high number points across the peak and good sensitivity for all analytes. Period 2 was the area of the method where these considerations were most important. A dwell time of 100 msecs was selected for all MRMs as all analytes gave sufficient data points across the peak. This period had the most analytes and a shorter dwell time allowed more cycles to occur. A dwell time of 125 msecs for these MRMs gave triangle shaped peaks for 3-hydroxyphenazepam, metizolam and meclonazepam with data points across the peak of 7, 10 and 9 respectively. The time period details for this method are shown in Table 42.

Table 42: Urine method mass spectrometer time periods

Time period					
1 (Starts at 0 Mins, Dwell time 125 msecs)		**2 (Starts at 13.5 mins, Dwell time 100 msecs)**		**3 (Starts at 19.11 mins, Dwell time 125 msecs)**	
	Data points		**Data points**		**Data points**
Pyrazolam	31	Alprazolam	15	Phenazepam	66
Nifoxipam	42	Flubromazolam	15	Diazepam	55
Chlordiazepoxide	36	Deschloroetizolam	15	Diclazepam	66
Clonazolam	25	Metizolam	14	Diazepam-D5	54
Nitrazepam	30	Oxazepam	18		
7-Aminoflunitrazepam-D7	35	Lorazepam	20		
		Meclonazepam	14		
		Temazepam	18		
		3OH-phenazepam	11		
		Etizolam	19		
		Lormetazepam	18		
		Flubromazepam	16		
		Desmethyldiazepam	17		
		Delorazepam	17		
		Lorazepam-D4	20		

4.5.1.3. Sample extraction

A LLE was chosen for the urine samples as a high throughput method was required for analysing all the samples - a cost effective and fast method was required, especially as a hydrolysis step was required. LLE is also a broad extraction technique that will allow all the analytes to be extracted which may be an issue with a SPE that is too selective.

The volume of extraction solvent *t*BME to be used was investigated and the results are detailed in Table 43. The results show that the %RE for QC1 at 0.75 mL *t*BME ranged from 9.5 to 62.8, %RE for QC2 at 0.75 mL *t*BME ranged from 11.7 to 69.0, %RE for QC1 at 1.75 mL *t*BME ranged from 6.7 to 58.6, %RE for QC2 at 1.75 mL *t*BME ranged from 12.5 to 86.4. From this experiment 1.75 mL *t*BME gives better recovery for QC2, the higher concentration (*p*-value =>0.05), 0.75 mL gives better recovery for QC1 (*p*-value=>0.05). The volume selected for the final extraction was 1.75 mL, the samples are urine and the benzodiazepine concentrations are typically higher in urine than in blood.

Table 43: Urine method – comparison of tBME used in the extraction process

Vol. of *t*MTBE (mL)	%RE				%ME			
	0.75 QC 1	**0.75 QC 2**	**1.75 QC 1**	**1.75 QC 2**	**0.75 QC 1**	**0.75 QC 2**	**1.75 QC 1**	**1.75 QC 2**
Drug								
Pyrazolam	9.5	11.7	6.7	12.5	93.5	94.6	92.1	99.6
Nifoxipam	40.1	43.6	36.4	51.6	93.5	94.0	89.1	98.8
Chlordiazepoxide	51.6	58.4	49.5	68.6	88.4	88.3	85.8	90.5
Clonazolam	33.6	36.9	26.8	44.5	91.6	89.5	90.6	92.8
Nitrazepam	60.5	67.8	52.5	76.1	85.2	84.6	77.5	87.7
Alprazolam	40.7	49.8	33.8	61.5	90.0	94.8	88.3	99.9
Flubromazolam	43.2	52.4	36.6	65.9	92.0	90.9	90.3	97.0
Lorazepam	57.8	64.5	52.0	81.6	97.1	100.2	93.9	106.5
Deschloroetizolam	46.6	55.3	39.8	64.1	88.6	90.1	88.9	94.2
Etizolam	44.0	51.3	37.0	64.4	86.6	89.1	82.2	93.3
Oxazepam	57.7	65.5	53.0	79.0	100.8	103.0	99.1	110.5
Meclonazepam	56.2	63.1	52.9	78.5	89.2	94.0	89.2	99.1
Temazepam	61.1	64.5	56.2	78.5	99.3	96.6	95.1	101.1
3OH-phenazepam	55.4	62.5	49.4	79.8	100.7	96.2	95.4	102.8
Metizolam	48.9	60.6	43.9	72.4	89.3	96.7	86.3	101.9
Lormetazepam	59.7	69.0	54.1	86.4	97.2	100.6	95.4	108.8
Flubromazepam	59.2	67.9	54.8	82.4	87.3	91.8	82.7	96.6
Delorazepam	57.0	64.1	52.7	81.9	94.1	96.8	90.8	103.6
DMD	62.8	68.5	58.6	75.9	91.2	92.9	89.2	94.4
Phenazepam	56.6	62.3	53.4	81.8	96.9	96.2	92.4	100.2
Diclazepam	51.8	58.7	48.2	73.9	85.2	89.3	84.1	93.8
Diazepam	57.7	64.6	55.2	74.0	83.9	85.6	81.5	88.4

The final urine LLE procedure utilised in this study is detailed in the flow diagram in Figure 40.

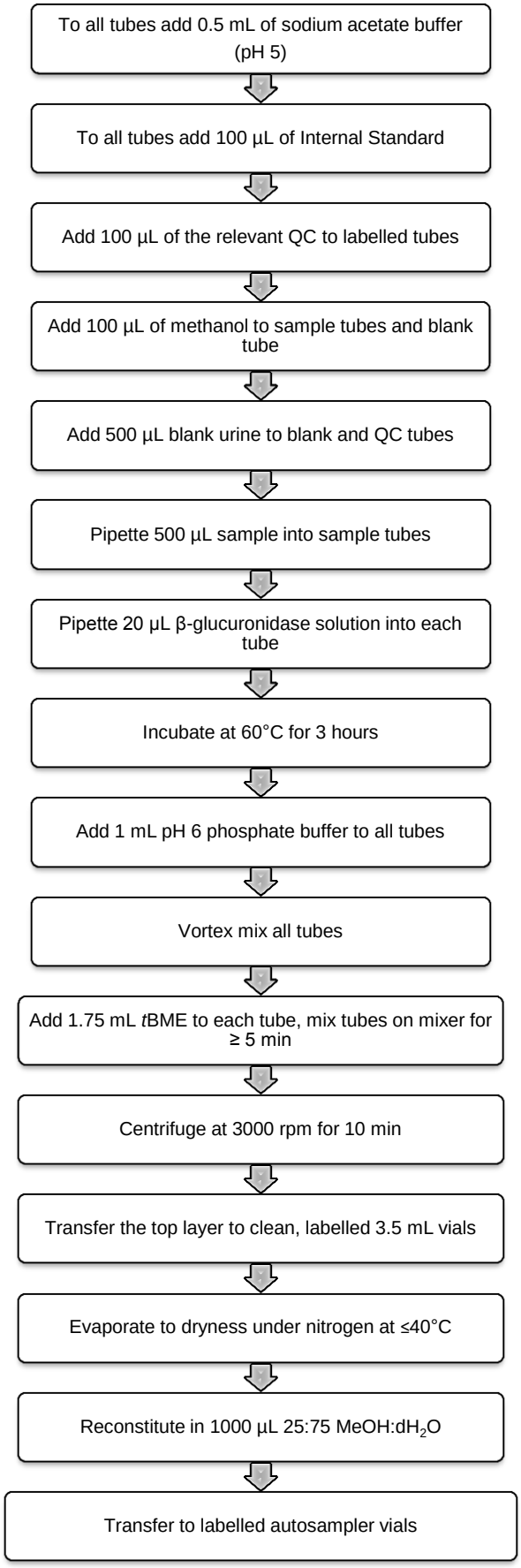

Figure 40: Urine method final LLE procedure

4.5.1.4. Hydrolysis step optimisation

Hydrolysis of the benzodiazepines is required when urine analysis is carried out due to the conjugation resulting from metabolism.

The three real urine samples used in this beta-glucuronidase optimisation study were positive for oxazepam, temazepam, desmethyldiazepam and lorazepam. Sample 2 and 3 were also positive for diazepam. Sample 1 was negative for diazepam. Table 44 shows the results of the beta-glucuronidase volume comparison. In most instances reducing the volume of the beta-glucuronidase had little impact on the peak area and at times improved it. There was an improvement in the oxazepam peak areas when 20 µL was used however there was a decrease in desmethyldiazepam. Due to these results it was concluded that 20 µL was sufficient to use for this method. The beta-glucuronidase used for the hydrolysis was ≥100,000 Fishman units /mL. One Fishman unit is commonly defined as the amount of enzyme required to liberate 1 µg of phenolphthalein from its glucuronide in 1 mL of urine per hour at 37°C at pH5. As the beta-glucuronidase used was ≥100,000 Fishman units /mL therefore there is ≥2,000 Fishman units in the 20 µL used in the urine extraction (≥4,000 in the 40 µL used in this comparison experiment). A study into the hydrolysis of benzodiazepines concluded that 5,000 Fishman units per 1 mL of urine at pH4.5 at 56°C for 2 hours was optimal. (Meatherall, 1994)

Table 44: Urine method - volume of beta-glucuronidase comparison (20 µL vs 40 µL) for the hydrolysis of benzodiazepines

	Oxazepam peak area (cps)		**Temazepam peak area (cps)**		**Desmethyldiazepam peak area (cps)**		**Diazepam peak area (cps)**		**Lorazepam peak area (cps)**	
Vol. of beta-glucuronidase (µL)	**20**	**40**	**20**	**40**	**20**	**40**	**20**	**40**	**20**	**40**
Sample 1	872174	674772	337311	278996	20030	21363	-	-	239444	183033
	783971	877893	317175	364408	20342	27801	-	-	213957	236641
Sample 2	7769134	7426079	11094760	11082305	1558520	1710703	81116	88430	9416	10087
	7876801	6975532	11541697	9945216	1597442	1455443	79535	76063	10674	8334
Sample 3	3020322	2828944	7968798	7994662	509057	669618	18994	20289	16895	15937
	3006728	2951060	7947879	8409910	544006	764595	18263	19150	16847	16332

4.5.2. Method validation

4.5.2.1. Limit of detection

Using the s/n ratio method all analytes had a LOD of 2 ng/mL (0.002 mg/L) with the exception of nifoxipam, lorazepam and 3-hydroxyphenazepam. Nifoxipam, lorazepam and 3-hydroxyphenazepam are all detected above the s/n noise criterion of 3 at 6 ng/mL (0.006 mg/L). The s/n ratio for lorazepam at 6 ng/mL is still low at 3.4 for the QT MRM, however the s/n ratio for the QL MRM is 80 at 6 ng/mL, which confirms it is detectable. The s/n results for the LOD experiment are shown in Table 45.

Table 45: Urine method LOD signal-to-noise ratio results

	s/n			
Analyte	**8 ng/mL**	**6 ng/mL**	**4 ng/mL**	**2 ng/mL**
Pyrazolam	73.5	25.5	33.5	25.5
Nifoxipam	15.5	11.0	0.0	0.0
Chlordiazepoxide	244.5	132.5	96.0	46.5
Clonazolam	39.5	35.5	15.0	24.5
Nitrazepam	204.5	129.5	44.5	26.2
Alprazolam	135.8	54.0	49.8	56.0
Flubromazolam	33.9	49.3	20.8	4.9
Lorazepam	15.0	3.4	3.0	0.0
Deschloroetizolam	118.8	25.8	31.4	84.0
Etizolam	570.5	121.2	120.2	239.5
Oxazepam	69.5	64.2	20.2	18.5
Meclonazepam	57.5	72.5	47.5	14.5
Temazepam	159.8	125.5	51.8	50.8
3OH-Phenazepam	31.5	13.5	1.0	0.0
Metizolam	723.5	295.5	32.5	137.5
Lormetazepam	148.5	94.0	16.2	24.0
Flubromazepam	68.5	37.5	6.5	5.5
Phenazepam	49.0	38.5	28.5	6.2
Diclazepam	63.0	45.9	22.5	19.8
Diazepam	119.8	89.5	55.5	33.6
Delorazepam	52.5	91.5	16.5	21.5
Desmethyldiazepam	138.5	33.5	19.5	21.0

4.5.2.2. Specificity

Table 46 shows the drugs/metabolites and internal standards injected as unextracted solutions individually down the LC-MS/MS method and which MRM transition a peak was present in. The table also shows the analytes present in the method, their retention time and the peak area from extracted low QC to use as a comparison. The crosses in the “Transitions with a response column” represent

the absence of a peak. Clonazepam is on the very edge of the nifoxipam transition, far from the retention time of interest. Deschloroetizolam and alprazolam interfere with each other with deschloroetizolam eluting first, the QT/QL ratio can be used to tell these analytes apart. Lorazepam-D4's presence in the alprazolam, deschloroetizolam and lormetazepam transition gave low peak areas less than 9000 for lormetazepam and less than 4000 for alprazolam and deschloroetizolam. It was expected the deuterated drug standards would be present near their drug counterpart with a slightly offset retention time. This experiment shows that while benzodiazepines may exhibit peaks in each others' transitions, this is not an issue as long as there is a reference standard for comparison and that the interferent peaks are resolved from the peak of interest.

Table 46: Urine method interference experiment results

Analytes in method	tR	Peak Area low QC	Individual drug injected	Transitions with a response	Peak Area	tR
7-Aminoflunitrazepam-D7	3.24	914578	Lorazepam-D4	Alprazolam QT	3789	15.59
Pyrazolam	6.64	30216		Deschloroetizolam QT+QL-jaggy peak	1523	15.60
Nifoxipam	8.60	32900	Diazepam-D5	x		
Chlordiazepoxide	6.70	239372	7-Aminoflunitrazepam-D7	x		
Clonazolam	12.10	69192	Metizolam	x		
Nitrazepam	12.02	199726	Pyrazolam	Clonazolam QT jaggy peak	995	6.55
Alprazolam	14.88	414059	Nifoxipam	x		
Flubromazolam	14.81	250535	Chlordiazepoxide	x		
Lorazepam	15.54	53922	Clonazolam	x		
Deschloroetizolam	14.70	920502	Nitrazepam	x		
Metizolam	14.65	642364	Flubromazepam	x		
Lorazepam-D4	15.39	899387	Flubromazolam	x		
Oxazepam	15.09	180929	Etizolam	x		
Meclonazepam	15.79	165295	Oxazepam	x		
Temazepam	16.44	787050	Temazepam	x		
3OH-phenazepam	16.54	45325	Lormetazepam	Lorazepam QT+QL	18661	16.04
Etizolam	17.22	713646		Lorazepam-D4	4780	16.00
Lormetazepam	17.97	407545	Lorazepam	Lorazepam-D4	881077	16.00
Flubromazepam	17.70	13650	DMD	x		
Desmethyldiazepam	17.77	298515	Phenazepam	x		
Delorazepam	18.57	193883	Diclazepam	x		
Phenazepam	19.88	59741	3OHPhenazepam	x		
Diazepam-D5	20.04	789624	Alprazolam	Deschloroetizolam QT+QL	184133	15.20
Diazepam	20.37	815308	Diazepam	Diazepam-D5	728344	20.24
Diclazepam	21.63	363439	Deschloroetizolam	Alprazolam QT+QL	107445	14.01
			Meclonazepam	x		
			Delorazepam	Diclazepam QT+QL	43071	22.2
			Flunitrazepam	x		
			Flurazepam	x		
			Clozapine	x		
			Midazolam	x		
			Clobazam	x		
			Prazepam	x		
			7Aminoflunitrazepam	x		
			Clonazepam	Nifoxipam QT	Cut off	≅ 13

4.5.2.3. Matrix effects, recovery and process efficiency

The matrix effects (%ME) for the low QC (0.015 mg/L) are displayed in Table 47 and the high QC (0.40 mg/L) in Table 48. The mean matrix effects for the low QC were all within the ±25% (criterion for %ME and <15% for precision. The Mean %ME for lorazepam, oxazepam and 3-hydroxyphenazepam all showed some enhancement at the high QC with a mean of 31, 34 and 28% respectively. This is higher than the criteria of 25%. All 10 sources of urine for these three analytes gave a %ME greater than 25%. These three analytes also elute within 1.5 minute of each other at around 15.00 to 16.50 minutes, improving the chromatographic conditions may improve the results for these analytes.

Table 47: Urine method validation - ME results at 0.015 mg/L

Drug QT %ME 0.015 mg/L	Overall Mean %ME (n=10)	Overall %CV (n=10)
Pyrazolam	2	4.4
Nifoxipam	11	4.2
Chlordiazepoxide	-10	4.2
Clonazolam	3	3.2
Nitrazepam	9	3.6
Alprazolam	0	5.1
Flubromazolam	-6	4.6
Lorazepam	17	4.6
Deschloroetizolam	0	3.1
Etizolam	4	3.6
Oxazepam	19	8.7
Meclonazepam	16	9.8
Temazepam	11	7.6
3OH-Phenazepam	19	5.2
Metizolam	5	3.3
Lormetazepam	12	4.8
Flubromazepam	14	8.6
Phenazepam	11	5.8
Diclazepam	1	2.0
Diazepam	1	3.3
Delorazepam	-11	3.8
Desmethyldiazepam	14	9.4

Table 48: Urine method validation-ME results at 0.40 mg/L

Drug QT %ME 0.04 mg/L	Overall Mean (n=10)	Overall %CV (n=10)
Pyrazolam	7	1.6
Nifoxipam	12	3.3
Chlordiazepoxide	3	1.7
Clonazolam	9	2.3
Nitrazepam	14	2.4
Alprazolam	19	0.9
Flubromazolam	2	1.9
Lorazepam	31	2.9
Deschloroetizolam	4	1.3
Etizolam	20	1.4
Oxazepam	34	5.0
Meclonazepam	14	11
Temazepam	15	4.0
3OH-Phenazepam	28	4.3
Metizolam	4	2.0
Lormetazepam	16	3.6
Flubromazepam	25	4.9
Phenazepam	16	3.5
Diclazepam	21	2.0
Diazepam	8	2.0
Delorazepam	22	5.7
Desmethyldiazepam	13	7.2

The %RE and %PE for both QCs are displayed in Table 49. Pyrazolam has considerably lower %RE and %PE compared to the other analytes. The %RE and %PE data for the internal standards in the urine method are shown in Table 50. 7-aminoflunitrazepam-D7 had the lowest mean %RE and %PE at 30% for both. This is adequate as no issues with the sensitivity were observed throughout the use of this method. The deuterated lorazepam and diazepam gave comparable results to their non-deuterated counterparts as expected.

Table 49: Urine method validation - mean percentage recovery (%RE) and process efficiency (%PE) for both low and high QC

	Low QC 0.015 mg/L		High QC 0.40 mg/L	
Drug QT	**%RE mean (n=10) (%CV)**	**%PE mean (n=10) (%CV)**	**%RE mean (n=10) (%CV)**	**%PE mean (n=10) (%CV)**
Alprazolam	34 (26.1)	34 (27.2)	43 (18.0)	52 (18.4)
Flubromazolam	39 (18.4)	37 (20.0)	48 (18.0)	49 (17.6)
Lorazepam	45 (28.3)	53 (27.8)	53 (21.0)	70 (19.3)
Deschloroetizolam	28 (20.9)	38 (22.1)	49 (17.9)	51 (18.2)
Etizolam	41 (21.0)	43 (22.7)	51 (18.0)	43 (22.7)
Oxazepam	47 (28.7)	56 (25.1)	47 (28.7)	56 (25.1)
Meclonazepam	47 (35.6)	53 (33.3)	60 (27.4)	68 (22.7)
Temazepam	54 (20.2)	60 (17.0)	60 (20.5)	69 (18.4)
3OH-phenazepam	48 (23.0)	57 (21.7)	52 (22.2)	67 (20.5)
Metizolam	46 (15.7)	48 (14.9)	57 (17.6)	60 (17.5)
Lormetazepam	54 (19.8)	61 (17.8)	58 (20.1)	67 (19.5)
Flubromazepam	48 (26.7)	55 (25.4)	59 (21.3)	73 (18.8)
Delorazepam	49 (25.5)	42 (22.9)	60 (10.7)	70 (19.5)
Pyrazolam	8 (17.5)	4 (18.5)	8 (20.6)	9 (21.1)
Nifoxipam	43 (25.3)	47 (24.5)	49 (18.8)	55 (18.3)
Chlordiazepoxide	48 (22.7)	43 (24.0)	61 (14.3)	63 (14.4)
Clonazepam	32 (23.3)	38 (22.1)	33 (20.8)	36 (21.5)
Nitrazepam	48 (25.3)	43 (22.7)	60 (17.9)	69 (18.2)
Desmethyldiazepam	50 (29.0)	56 (25.0)	61 (21.8)	68 (18.1)
Phenazepam	48 (26.7)	53 (23.8)	57 (19.9)	65 (18.6)
Diclazepam	50 (22.2)	50 (22.2)	59 (17.2)	72 (16.9)
Diazepam	49 (21.9)	50 (21.9)	63 (15.4)	68 (14.9)

Table 50: Urine method validation - mean percentage recovery (%RE) and process efficiency (%PE) for internal standards

Drug QT	%RE mean (n=10) (%CV)	%PE mean (n=10) (%CV)
7-aminoflunitrazepam-D7	30 (23.1)	30 (23.1)
Lorazepam-D4	48 (25.3)	52 (24.2)
Diazepam-D5	53 (22.0)	51 (21.6)

4.5.2.4. Carryover

Carryover is assessed by the absence or presence of peaks in the injections of drug-free reconstitution solution directly following injections of a high concentration of the analytes of interest. There were no peaks in the drug-free reconstitution solution injection that followed three injections of the extracted stock solution containing all analytes at a concentration of 4 mg/L, see Figure 41. The increase in the instrument noise in period 2 compared to period 1 and 3 is clearly demonstrated in Figure 41. This is due to period 2 having more analytes, a lower dwell time and less cycles for the MRMs contained in that period. This is discussed in section 4.5.1.2 of this chapter.

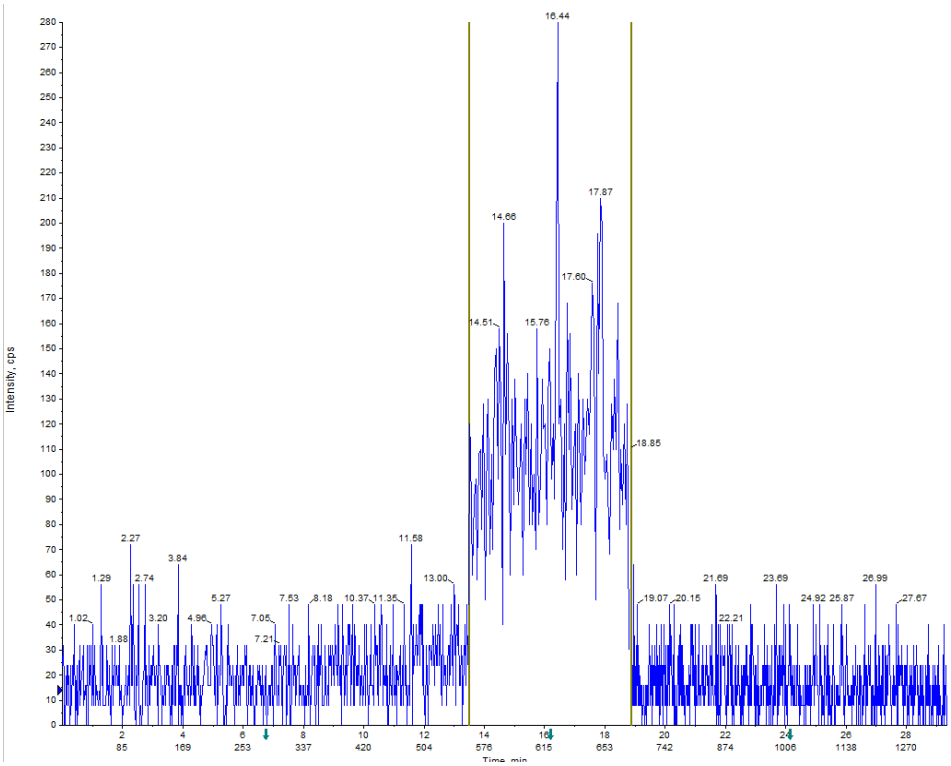

Figure 41: Example chromatogram of the absence of peaks after injections of 4 mg/L, demonstrating the lack of carryover.

4.5.2.5. Autosampler stability

The autosampler stability test results are shown in Figures 42 to 44 for the low (0.015 mg/L) QC concentration and Figure 45 to 47 for the high (0.150 mg/L) QC concentration. Each concentration was injected in triplicate every 4 hours, the average peak area was plotted on the graphs displayed in Figures 42 to 47. All compounds were still above 20% (represented by red line in the charts) of t0 after 36 hours within the autosampler at 16-24 °C, this demonstrates that up to 36 hours queued waiting to be injected is not an issue but can be more detrimental to some analytes than others within the method. Most analytes stayed fairly unchanged, some such as nifoxipam, flubromazepam and desmethyldiazepam showed a downward trend suggesting the stability was in decline. Nifoxipam is a nitro-benzodiazepines, which are chemically unstable particularly in bacteria contaminated samples such as post-mortem samples, it is recommended that preservative is used for the collection of samples potentially positive for nitro-benzodiazepines. (Robertson and Drummer, 1998; Levine, Blanke and Valentour, 1983) One stability study has shown that clonazolam, also a nitro-benzodiazepine

and nifoxipam are not stable in urine at -20°C and had degraded up to 38 and 40% respectively over the course of seven months. A low pH in urine can degrade nitro-benzodiazepines due to ring-opening. (Bergstrand, Helander and Beck, 2016) Without the correct collection and storage it may be extremely challanging to detect nitro-benzodiazepines in post-mortem blood or urine due to these stabilty issues confounded by the lack of available certified reference materials for the designer nitro-benzodiazepine metabolites.

Some analytes such as chlordiazepoxide and clonazolam have shown an upward trend. This may also occur if there is slight evaporation of the solvent leading to a more concentrated solution, the autosampler is not temperature controlled however the laboratory has a temperature monitoring system in place and is maintained.

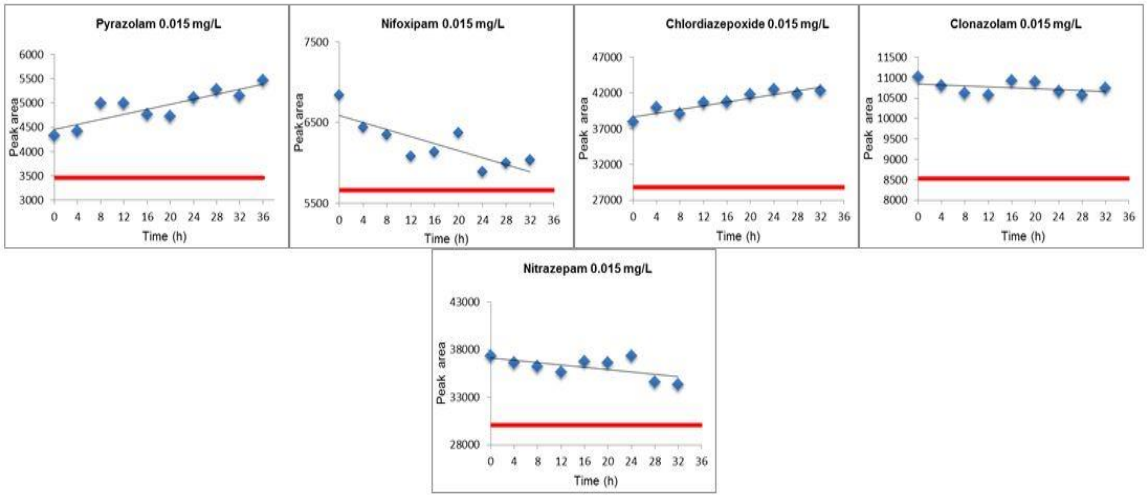

Figure 42: Urine method validation - Autosampler stability for first time period analytes at a concentration of 0.015 mg/L over 36 hours.

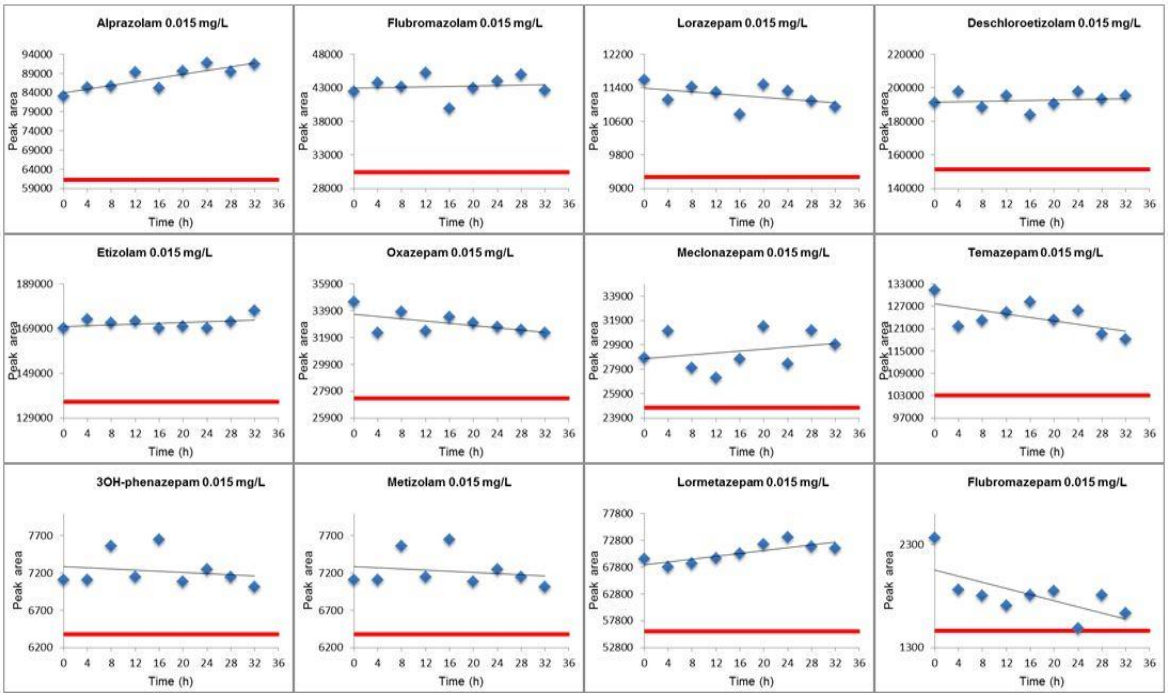

Figure 43: Urine method validation - Autosampler stability for second time period analytes at a concentration of 0.015 mg/L over 36 hours.

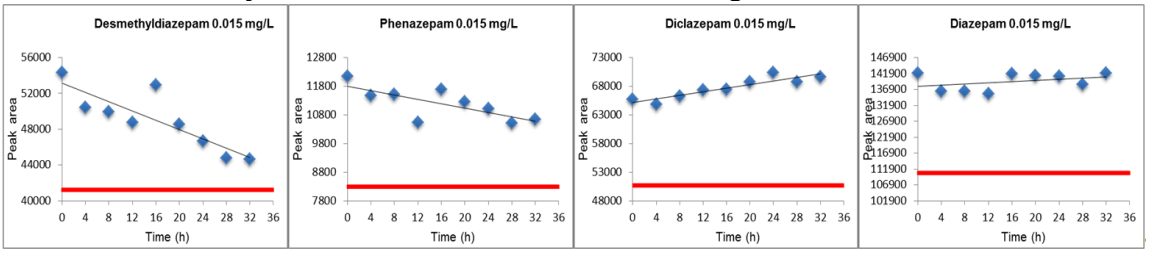

Figure 44: Urine method validation - Autosampler stability for third time period analytes at a concentration of 0.015 mg/L over 36 hours

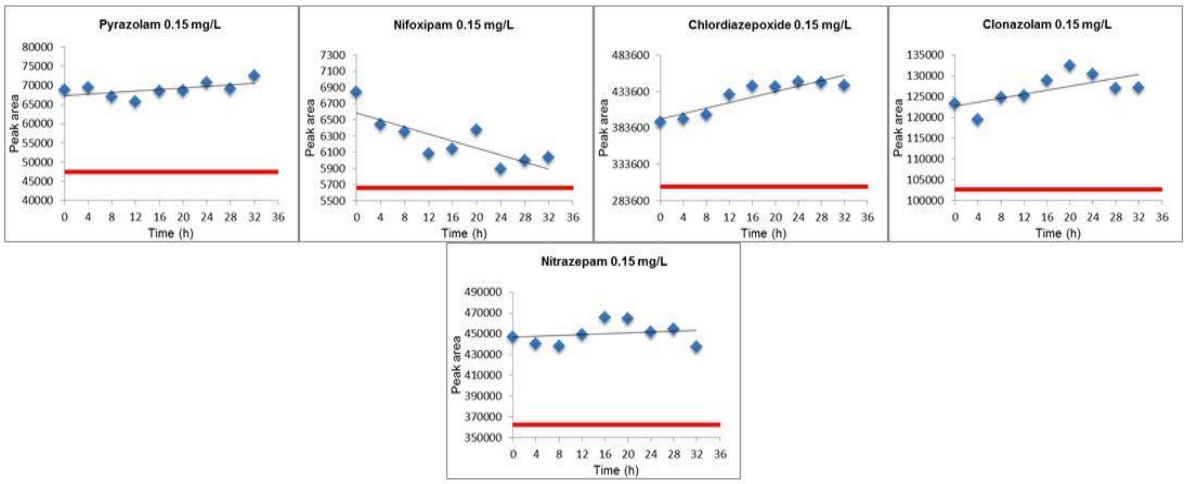

Figure 45: Urine method validation - Autosampler stability for first time period analytes at a concentration of 0.15 mg/L over 36 hours.

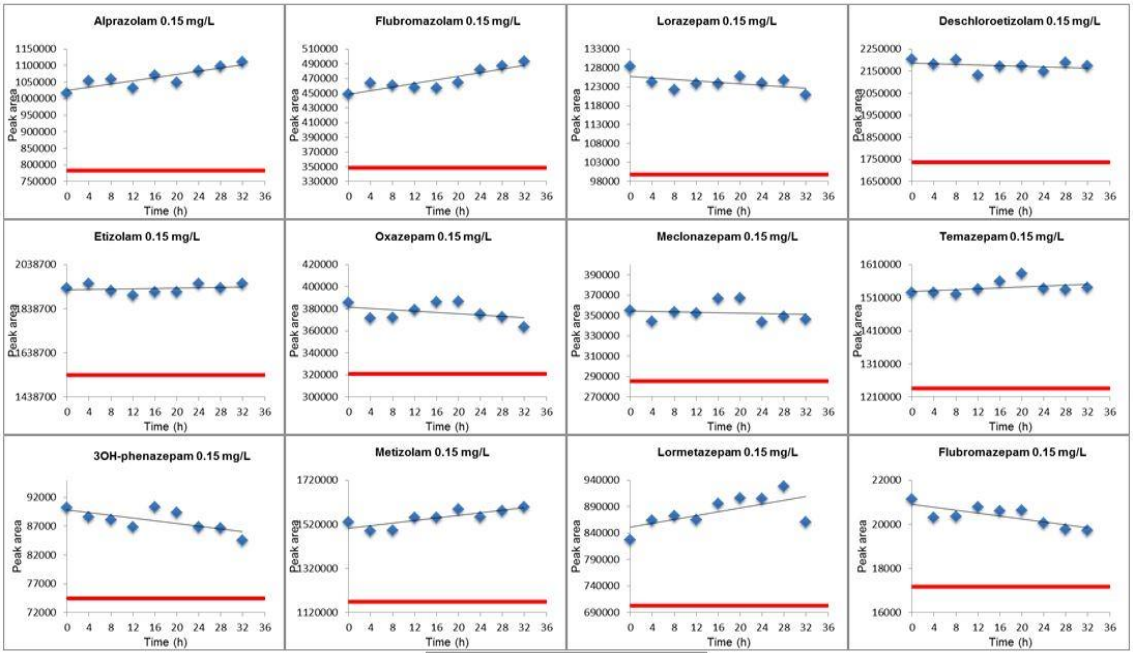

Figure 46: Urine method validation - Autosampler stability for second time period analytes at a concentration of 0.15 mg/L over 36 hours.

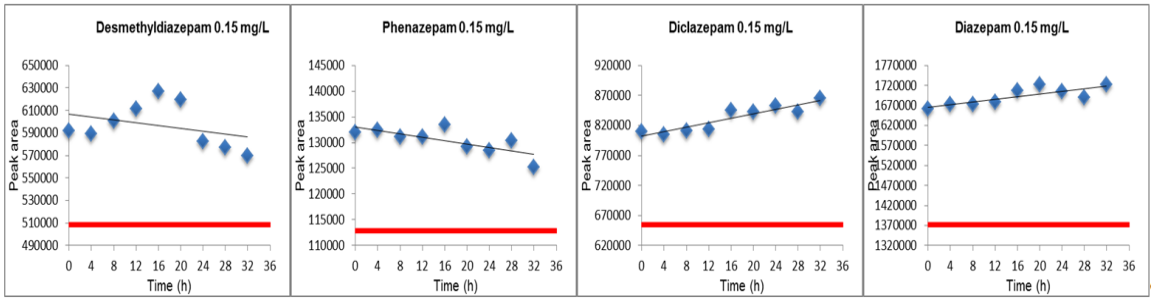

Figure 47: Urine method validation - Autosampler stability for third time period analytes at a concentration of 0.15 mg/L over 36 hours.

4.6. Conclusion

This method was developed, validated and found to be suitable for the qualitative analysis of urine samples for 22 analytes including both traditional and designer benzodiazepines and metabolites. The method MS/MS parameters were optimised for the 22 analytes and 22 gradient programmes were tested to achieve good chromatographic resolution with set MS time periods created to achieve improved Gaussian peak shapes with sufficient counts across the peak (>10). The two different volumes of extraction solvent were assessed before the final volume of 1.75 mL was selected. The volume of beta-glucuronidase used in each tube was reduced by half, after an investigation showed there was adequate hydrolysis activity at 20 µL.

The analytes were detectable down to 2 ng/mL with the exception of nifoxipam, lorazepam and 3-hydroxyphenazepam, which were detectable at 6 ng/mL. Benzodiazepine specificity for the method was tested and interferents were found to be minimal; alprazolam and deschloroetizolam gave the most interference with each other but can be identified by their QT/QL ratio. Matrix effects were found to be acceptable for all analytes at the low concentration and within the ±25% criterion for %ME and <15% for precision with slight enhancement for three analytes (lorazepam, oxazepam and 3-hydroxyphenazepam) at the higher concentration. Recovery was suitable with the exception of pyrazolam however it had a sufficient LOD of 2 ng/mL however, due to the sub-optimal recovery this method may not a be suitable to analyse pyrazolam in urine and caution should be given to any pyrazolam positive samples (or suspected pyrazolam positive samples) and subsequently these should be confirmed using an additional method. No carryover was observed in the drug free reconstitution solution after 4 mg/L was injected. All analytes were stable in the autosampler stability up to 36 hours at 16-24 °C.

5. Drug Court and Forensic Directorate samples

5.1. Introduction

The use of NPS is often undetected by conventional methods of testing for drugs of abuse such as immunoassay or a defined drug panel. Chapter 2 demonstrated how the Immunalysis® Benzodiazepine ELISA kit immunoassay is effective at detecting six of the benzodiazepines explored in this study however this may not apply to all commercially available immunoassay kits and may not be the case with dip stick tests. Chapter 2 evaluated blood samples and not urine where the glucuronides may cause detection issues. There is a concern for frontline drug treatment services that they may miss cases of drug misuse and therefore not provide the most effective advice or treatment. As has always been the case with illicitly made drugs, the user cannot be sure of what they are taking; with the proliferation of NPS this risk has become ever present. The inability to routinely detect these substances in a treatment setting means the real extent of NPS in these sub-populations is unknown. Urine analysis has the advantage of being less invasive than blood and yields a high volume sample. The window of detection in urine is longer than in blood, especially when metabolites are also monitored, for example, studies found that pyrazolam and delorazepam (metabolite of diclazepam as well as a drug in its own right) can both be detected in urine up to six days after a single administration of pyrazolam and diclazepam. (Moosmann *et al.*, 2013b; Moosmann, Bisel and Auwärter, 2014) This longer window of detection is useful for testing in populations where abstinence from drugs is particularly important, such as workplace drug testing, in offenders or in patients where drug use may be especially damaging, such as benzodiazepine use in individuals with severe mental health disorders. Urine samples have to be collected under the right conditions such as direct observation as they can be easy to substitute for a drug-free sample or to adulterate by diluting.

This chapter describes the targeted testing of designer benzodiazepines in two at-risk populations, Scottish Drug Court participants and Forensic Psychiatric patients.

5.1.1. Scottish Drug Court participants

The Scottish Drug Court (SDC) is a service that aims to reduce drug misuse, and the offences associated with drug misuse, such as theft, to fund a drug habit. The court orders a Drug Treatment and Testing Order (DTTO) as an alternative to

prison and imposes an obligation to be treated for drug abuse and to commit to change criminal behaviour. (NHS Greater Glasgow and Clyde) The Scottish Drug Court (SDC) treats a weekly average of 75 individuals under a DTTO.

Drug testing is routinely undertaken by the Drug Court to ensure abstinence from illicit drug use as part of sentencing and treatment. A urinary immunoassay dipstick test (Alere ® Drug Screen Urine Test Strip) is employed, which covers the most commonly abused drug groups including benzodiazepines, opiates, cannabis, cocaine and amphetamines, however it is not designed to test for, and does not include, NPS.(Alere Toxicology Point of Care Cut offs) The immunoassay results obtained by the drug court were not shared with the author and therefore were not available to compare with this studies results.

5.1.2. Forensic Directorate psychiatric patients

Approximately 70% of patients treated by NHS Greater Glasgow and Clyde Forensic Directorate (NHS GGC FD) have a primary diagnosis of schizophrenia although many have co-morbidity with a range of diagnoses' combined with a history of illicit drug use and alcohol abuse. Most patients are detained under either the Mental Health (Care and Treatment) Scotland Act (2003) or the Criminal Procedure (Scotland) Act 1995. It is a condition of the patient's suspension of detention or condition of discharge that they must not use drugs and alcohol.

5.2. Aim

To evaluate the use of benzodiazepines including designer benzodiazepines in individual treatment programmes under the jurisdiction of the SDC system, and how this compares to self-assessment questionnaires; and also in patients undergoing treatment by the NHS GGC FD. Hypothesis: The samples from individuals from the SDC and patients from NHS GGC FD will have a higer frequency of positive results for benzodiazepines than noted on their questionnaires.

5.3. Ethical considerations

The SDC work was considered as 'service development' and as such NHS ethical approval was not deemed necessary. Ethical approval was sought and granted from the University of Glasgow, College of Medical, Veterinary & Life Sciences Research Ethics Committee (application number 200140101), (see appendix 11.2)

Ethical approval for the Forensic Directorate cohort was sought and granted from the NHS Greater Glasgow and Clyde, West of Scotland Research Ethics Service (WoSRES) (Application number 15/WS/0263), (see appendix 11.3)

5.4. Method and materials

5.4.1. Materials

The materials used are detailed in section 4.3.1 of chapter 4.

5.4.2. Extraction Method

Urine samples were extracted using the LLE extraction method detailed in section 4.5.1.3 of chapter 4.

5.4.3. Instrumentation and method

The extracts were analysed using the LC-MS/MS method detailed in section 4.3.4 of chapter 4. This method was employed as a qualitative method with cut-off concentrations. Table 51 details the analytes included in the method. The cut-off used for this method was 0.015 mg/L (15 ng/mL) as this concentration was tested for the matrix effects and recovery (see section 4.4.2.3).

Table 51: Benzodiazepine compounds included in the LC-MS/MS method

Prescribed Benzodiazepines	Designer Benzodiazepines
Diazepam and metabolites (desmethyldiazepam, oxazepam and temazepam)	Diclazepam and metabolites (delorazepam, lorazepam and lormetazepam)
Chlordiazepoxide and metabolites (desmethyldiazepam and oxazepam)	Etizolam*
Nitrazepam	Alprazolam*
	Phenazepam*
	3-Hydroxyphenazepam
	Flubromazolam
	Pyrazolam
	Clonazolam
	Deschloroetizolam
	Meclonazepam
	Nifoxipam
	Metizolam

*As explained in section 1.5.1 in chapter 1, these are considered designer benzodiazepines for simplicity

5.4.4. Sample collection

5.4.4.1. Scottish Drug Court samples

Sample collection was carried out over one week each month (except December) between August 2015 and February 2016. Every participant who attended the Drug Court for urine testing was offered the opportunity to take part in the study. Participants were over 21 years of age, a history of problem drug use and a criminal record. The nurse in charge of the collection of their mandatory urine sample approached the participant and provided them with the study information sheet and the consent form (see appendices 11.3 and 11.4). Participants were reassured that the results of the analysis were anonymised and were not used in any legal proceedings or shown to the Sheriff overseeing the Court. The Nurse Team Leader was the only person with access to the signed consent form, which could be linked to the questionnaire (see appendix 1.8) and urine sample, this traceability was maintained as an individual could decide to withdraw from the study at any time. The schematic in Figure 48 was used to explain the process in the ethics application.

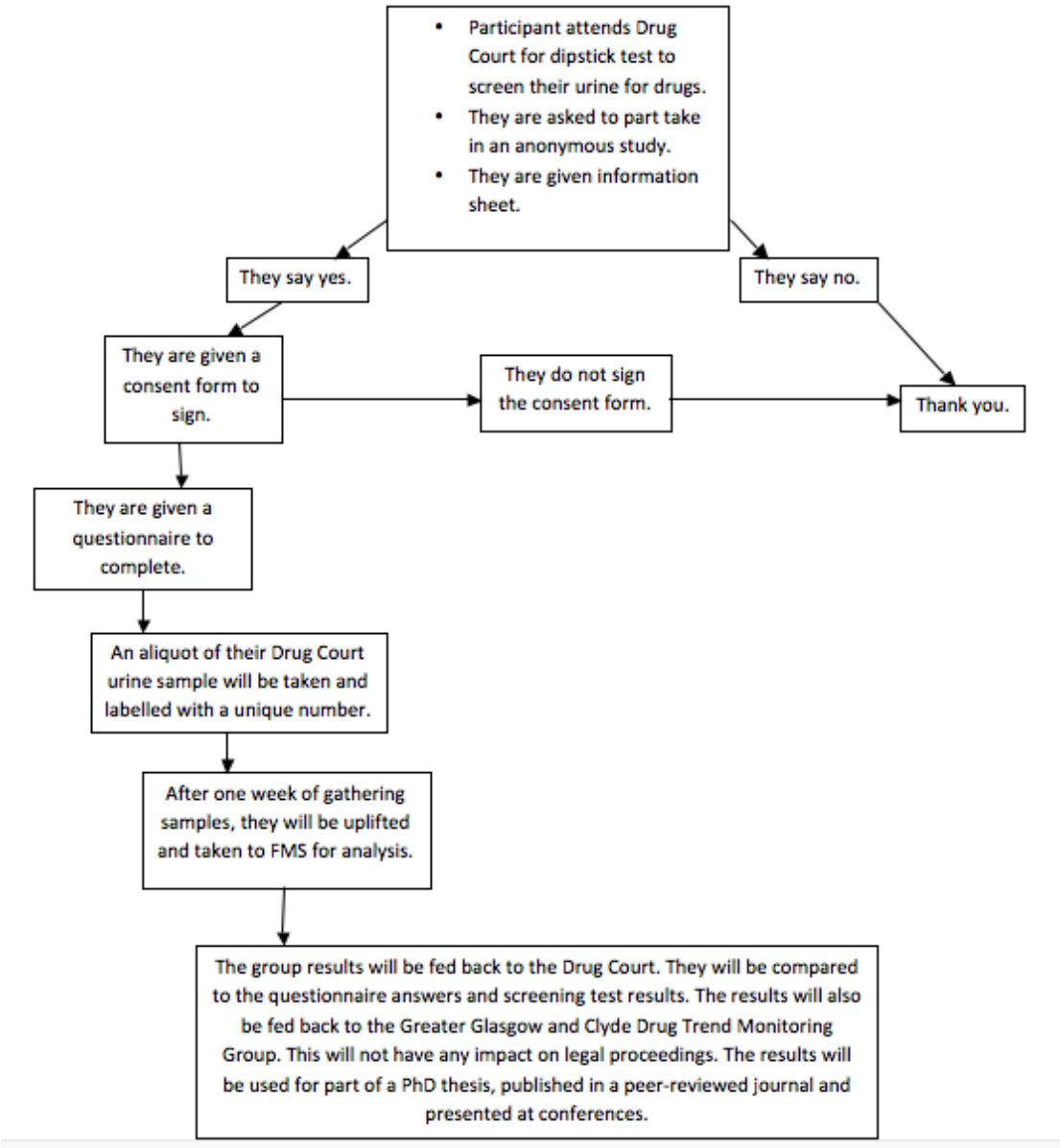

Figure 48: Flow chart describing the SDC sample process

The samples were refrigerated (2-8°C) after donation then taken to Forensic Medicine and Science (FMS) at the University of Glasgow. The Drug Court did not have a freezer, as they do not usually store any biological samples. The samples were frozen immediately at −20°C on receipt at FMS until they were analysed. The samples collected usually contained at least 10 mL of unpreserved urine or more.

The samples were labelled “DC 01” to “DC 73” and linked to their questionnaire using this numerical system. It was possible for individuals to give more than one sample on different weeks however it was not possible to link this or know where or if this occurred.

5.4.5. Data handling and statistical analysis

Once results had been generated in the Analyst software, they were copied onto a Microsoft® Excel® (version 14.7.3) spreadsheet and batch checked by a second toxicologist before any calculations were performed. The results copied include the peak areas of the analyte and the internal standard, the calculated values of the calibration standards, samples and QCs in mg/L and the datapath information. Microsoft® Excel® was then used to generate the charts and descriptive statistics within the results section. All personal data was kept anonymised. The anonymised questionnaire data was entered into a Microsoft® Excel® spreadsheet and kept on a secure drive to protect the data.

5.4.5.1. Forensic Directorate psychiatric samples

Urine samples were collected from January 2016 to November 2016 in FD facilities as part of the standard drug testing procedure. Participation is this study was voluntary. Participants were provided with an information sheet and a consent form (see appendices 11.5 and 11.6). NHS biomedical scientists carried out the initial testing procedure which includes splitting the collected urine sample into two separate aliquots; one is retained for independent testing if required whilst the other is used for the initial drug screen. The initial immunoassay drug screen was conducted using an automated system called an Abbott Architect analyser. This testing system has a benzodiazepine cut-off of 200 ng/mL. (Abbott Architect, Benzodiazepine Manufacturer Kit Insert, 2018) No dip stick tests were used for these samples. Positive screened samples were confirmed at the same laboratory, and the remainder of the sample was then sent to FMS for the additional testing. Each urine sample was collected in 6 mL white-topped vacuette tubes, which contained no preservative. On average the samples contained 4 mL of urine. Positive screens were confirmed by GC-MS in the Queen Elizabeth University Hospital and were noted on the form sent to FMS. All anonymised samples and paperwork were transported to FMS by courier. Samples were labelled “FP 01” to “FP 95” and linked to their consent form using this numerical system.

All samples were stored at −20°C on receipt at FMS until they were analysed. The storage conditions prior to this are not known.

5.5. Results and discussion

5.5.1. Scottish Drug Court results

Urine samples were collected from 73 individuals in total under the supervision and treatment of a DTTO through the SDC system in Glasgow. A Drug Court nurse collected the samples as part of their mandatory drug testing, no additional sample was collected from the donor for this study. The samples were collected in 25 mL universal vials containing no preservative. Participants also completed a questionnaire about their licit and illicit drug use. Table 52 displays the collection dates.

Table 52: SDC collection weeks for urine samples

Week beginning	Samples collected
31/08/2015	DC01-DC25
28/09/2015	DC26-DC47
26/10/2015	DC48-DC51
23/11/2015	DC52-DC57
18/01/2016	DC58-DC67
15/02/2016	DC68-DC73

5.5.1.1. Questionnaire responses

Some basic demographic information was obtained from the questionnaire (see Appendix 11.8) responses. There were 64 samples from males, 4 from females and 5 samples with no sex detailed. According to the Drug Court nurse this is in keeping with the drug court system treating more men than women in general. The age range spanned from 24 to 65 years old. It is important to note that over the six-month collection period the same individual was able to participate more than once so 73 samples does not necessarily mean 73 different individuals. It was unknown if this had occurred, as the data was kept anonymised.

The participants were provided with a space to complete a questionnaire with regards to their drug use using free text opposed to a tick box therefore they used their own terms to report it. Every respondent mentioned that they had taken at least one drug in the week prior. Most participants named 2 drugs on their questionnaire (n=25). The graph in Figure 49 shows how many drugs participants mentioned.

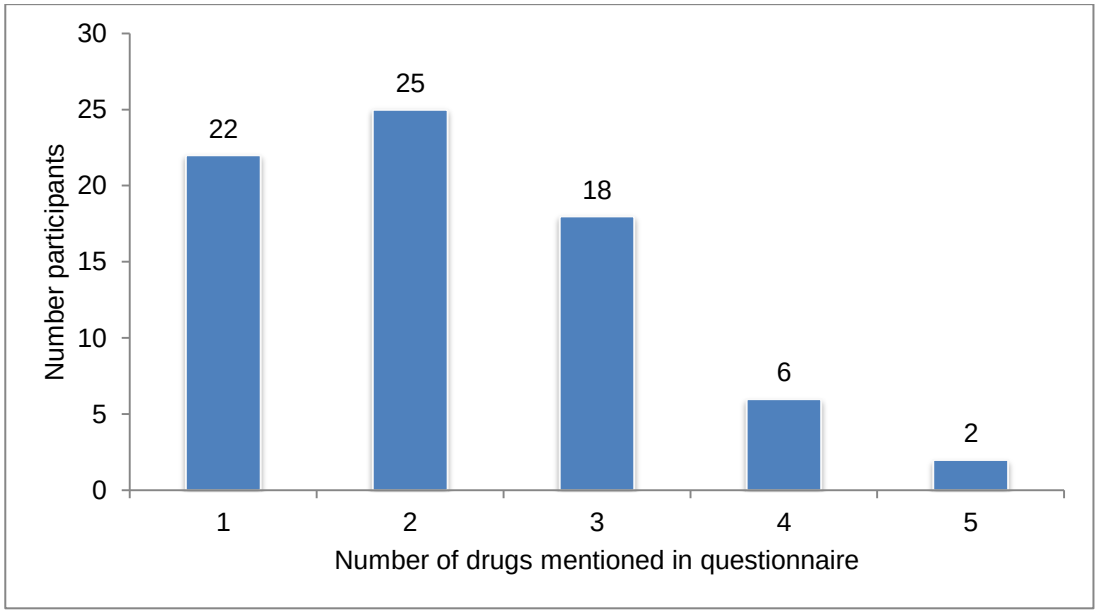

Figure 49: SDC number of drugs mentioned per questionnaire by participant

From the 73 questionnaires,”diazepam” or ”benzodiazepines” were mentioned 14 times (19%). Both were treated as one drug in this instance. There was no mention of any designer benzodiazepines or any brand name associated with them e.g. chillax. Figure 50 displays the non-benzodiazepine drugs mentioned by the participants in their questionnaire.

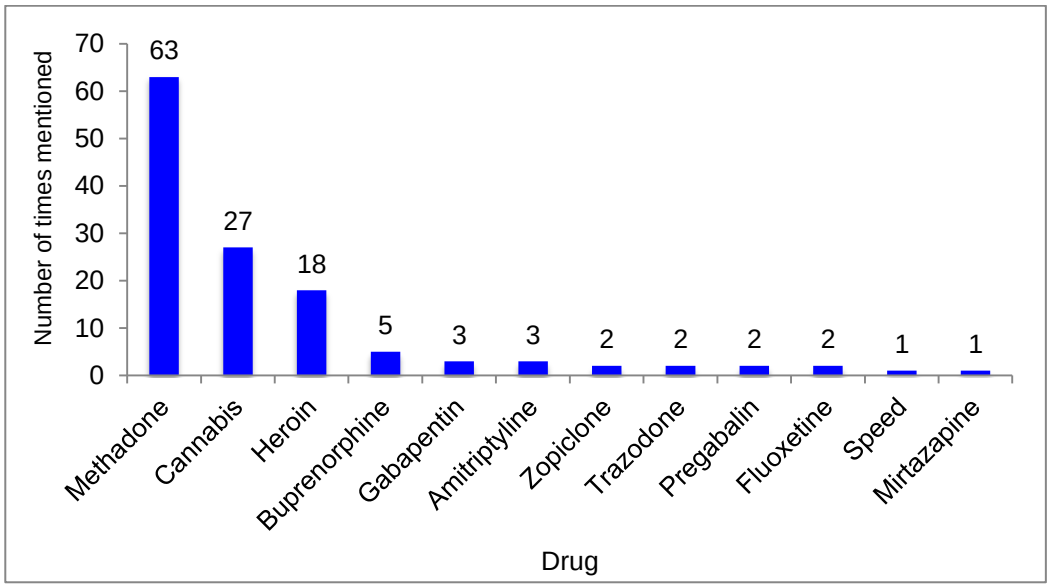

Figure 50: SDC non-benzodiazepine drugs mentioned in the questionnaires

According to a report published in June 2018 by the Information Services Division (ISD) heroin was the most reported illicit drug to the Scottish Drug Misuse Database (SDMD) in the month prior to their assessment at 52%, followed by cannabis at 32%, diazepam at 29% and cocaine/crack at 19%. The SDMD gathers data from individuals reporting to drug treatment services; the SDMD aim is to monitor drug use, identify trends and feedback to treatment services, therefore it deals with the same population as this study. Figure 51 displays the drugs reported to the SDMD over the years. (Information Services Division, Scottish Misuse Database, Overview of initial assessments for specialist drug treatment 2016/2017, 2018)

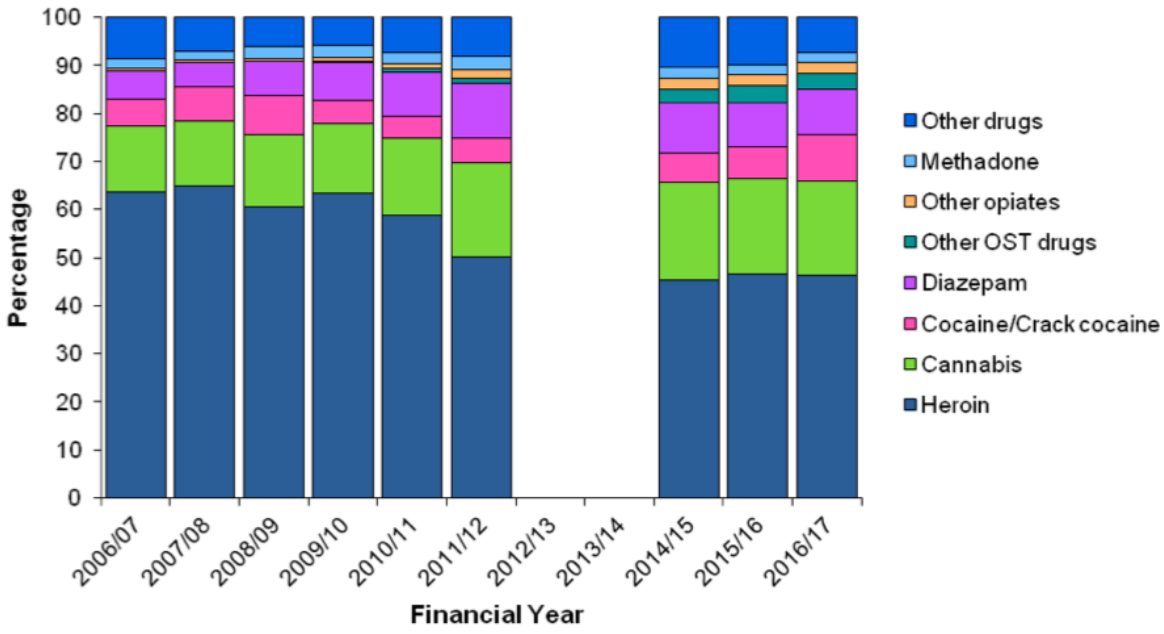

Figure 51: SDMD illicit drugs use reported over a 10 year period
(reproduced with permission from Public Health Scotland)

OST stands for Opioid Substituted Therapy. The data from 2012-2014 is missing due to data quality and completeness *issues.* (Information Services Division, Scottish Misuse Database, Overview of initial assessments for specialist drug treatment 2016/2017, 2018)

It should be noted that the methadone use reported in Figure 51 is illicit methadone use. The questionnaire used in this study asked for all drug use and they recorded their prescribed methadone use. The most common non-benzodiazepine drug mentioned in this study was methadone, which is only a small portion in the SDMD findings, heroin is the third most mentioned in this study

compared to first. Perhaps the participants in this study did not hesitate to mention of methadone as they were prescribed it but were more reserved about discussing heroin as this is breaking their DTTO. Both found cannabis to be the second most reported drug.

5.5.1.2. Scottish Drug Court laboratory results

The SDC urine samples were positive for 9 different benzodiazepine analytes.

Table 53 displays the number of positive urine samples for the benzodiazepine drugs tested. All other samples were negative for the benzodiazepines specified in Table 51. Concentrations are not reported, as this was a qualitative study.

Due to the complex nature of the benzodiazepine class of drugs, care must be taken when interpreting benzodiazepines detected in urine. Benzodiazepines often metabolise, or break down, to other active benzodiazepine drugs, e.g. diclazepam forms delorazepam, lorazepam and lormetazepam in the body. In addition to being active metabolites, these substances are drugs in their own right, and some can be formed from more than one parent drug e.g. delorazepam is a metabolite of both diclazepam and cloxazolam (not licensed for use in the UK, prescribed in Italy). (Manchester *et al.*, 2018; Moosmann, Bisel and Auwärter, 2014)

Diazepam is the most prescribed benzodiazepine worldwide and metabolises to three other active drugs, desmethyldiazepam, oxazepam and temazepam. Desmethyldiazepam is also a metabolite of six other benzodiazepine drugs. Diazepam and its metabolites were the most detected benzodiazepines in this study.

Table 53: SDC number of urine samples positive for benzodiazepines

Analyte	No. of Positives
Diazepam	15
Desmethyldiazepam	34
Temazepam	35
Oxazepam	40
Delorazepam	2
Lorazepam	5
Lormetazepam	2
Etizolam	1
Metizolam	1

The results showed 2 positives for delorazepam indicating diclazepam use, (Moosmann, Bisel and Auwärter, 2014) DC 41 and DC 72, see Table 54. In addition sample DC 41 was positive for a further 2 diclazepam metabolites

(lorazepam and lormetazepam). Lorazepam and lormetazepam are clinical drugs in Scotland however not commonly prescribed, especially compared to benzodiazepines such as diazepam, see section 1.3 in chapter 1. The combination of the drugs together also adds weight that they are the product of diclazepam metabolism rather than lorazepam and lormetazepam consumption. Sample DC 41 was collected from a 30-year-old male, his questionnaire does not mention any benzodiazepines; it states he had only taken methadone in the week prior to sample collection. It is reasonable to conclude that DC 72 and DC 41 have used diclazepam as they are positive for more than one of its metabolites. Diclazepam metabolism is discussed in section 1 .7 of chapter 1. The questionnaires for DC 41, DC 72 and DC 49 state they are all male and they had only taken methadone in the week prior to sample collection. DC 41 and DC 49 are positive for diazepam metabolites but not diazepam itself, which is not unusual in urine samples. (Temte *et al.*, 2018) DC 64 and DC 65 are positive for lorazepam only, which may be from the use of prescribed lorazepam, although this was not stated, or the use of diclazepam. DC 64 is a 36-year-old male who stated he had taken diazepam from a dealer as well as methadone and cannabis in the week prior to his sample collection. DC 65 is a 65-year-old male who states he had taken heroin and methadone in the week prior to his sample collection.

Table 54: NHS GDDC urine samples positive for diclazepam metabolites

Sample number	Age	Sex	Drug mentioned on questionnaire	Toxicology findings
DC 41	30	M	Methadone	Delorazepam Lorazepam Lormetazepam Oxazepam Temazepam Desmethyldiazepam
DC 49	34	M	Methadone	Lorazepam Lormetazepam Oxazepam Temazepam Desmethyldiazepam
DC 72	51	M	Methadone	Delorazepam Lorazepam Oxazepam Temazepam Desmethyldiazepam Diazepam

Sample DC 53 was positive for both etizolam and metizolam, see section 5.5.1.3 for an investigation into the metizolam positive samples.

Sample DC 53 was also positive for diazepam and all three metabolites. Sample DC 53 was collected from a 36-year-old male who stated in his questionnaire that he had taken ‘benzodiazepines’ in the form of 5 x 5mg street diazepam he had

purchased from a dealer. He states he took these the day before attending the Drug Court to provide his urine sample. DC 53 was the only sample positive for etizolam. This is unusual as etizolam has been shown to be a very popular drug in Scotland however no metabolites for etizolam were in the method and this will reduce detection. The half-life of etizolam is 3 to 7 hours (Høiseth, Tuv and Karinen, 2016) and is much shorter than diazepam and diclazepam (20-50 and 42 hours respectively) so therefore etizolam has a shorter window of detection compared to diazepam and diclazepam.

It is reasonable to speculate that all of the positive results for oxazepam, temazepam and desmethyldiazepam came from diazepam use as these are the major diazepam urinary metabolites and as discussed in 1.3 of chapter 1 diazepam is much more available than oxazepam. (Temte et al., 2018) The 15 positives for diazepam are also positive for all three diazepam metabolites.

In total there were 40 (55%) samples positive for benzodiazepines, all 40 were positive for more than one analyte tested. This is higher than the reported use on the questionnaires. As the result of the dip stick tests were never reported to the author, it is not possible to compare the results of this analysis to the dip stick results. This study shows that benzodiazepine use is common in this population and it is possible that individuals may be using designer benzodiazepines either in an attempt to evade detection by the immunoassay tests used in mandatory drug testing or inadvertently due to illicitly made “valium” tablets. (Kirby, 2016) The ability to evade detection is a concern for the healthcare workers in these settings however in this study the samples in which designer benzodiazepines detected were all positive for diazepam and therefore would test positive by immunoassay leaving this question not fully answered. The study is also voluntary and thereby users may have not agreed to take part if they had recently used designer benzodiazepines. The use of benzodiazepines is common amongst opioid users as they can be used to enhance the high or to self-medicate during withdrawals or insomnia. (Jones, Mogali and Comer, 2012)

5.5.1.3. Metizolam investigation

Metizolam appeared on the NPS market in 2015. (EMCDDA, Europol, EMCDDA-Europol 2015 Annual Report on the Implementation of Council Decision 2005/387/JHA, 2016) It is an analog of etizolam and differs only structurally in that it has one less methyl group, see Figure 52.

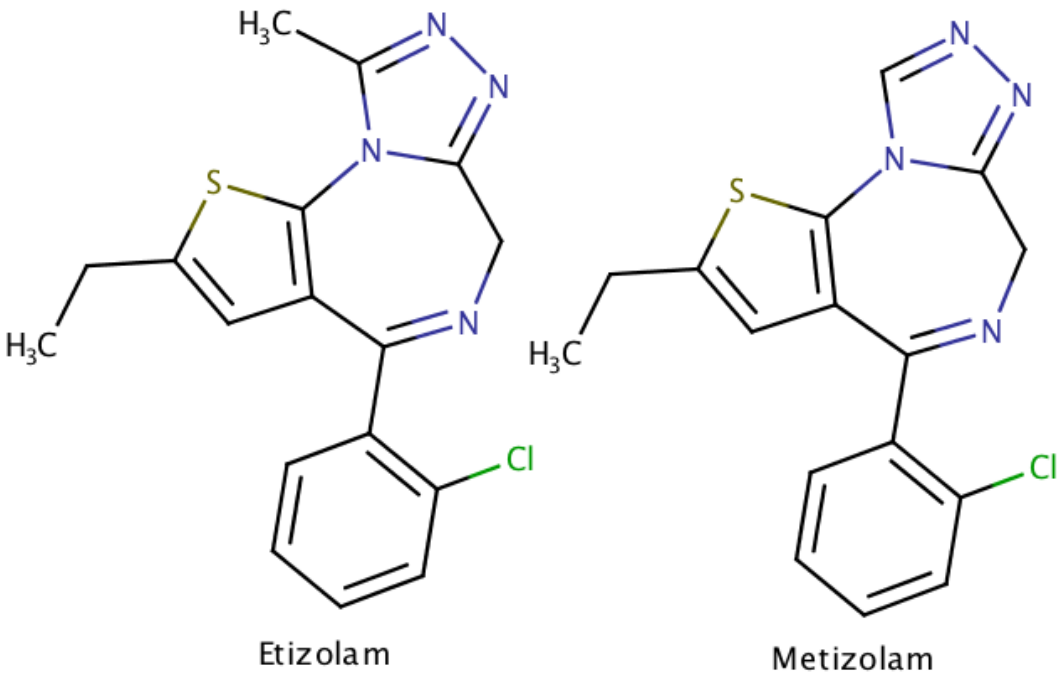

Figure 52:Chemical structure of etizolam and metizolam

Due to the lack of published research it is difficult to determine the source of metizolam in the urine samples; the individual may have ingested both etizolam and metizolam or metizolam may be a degradation product of etizolam however it is unknown what would be causing this if this was the case. All metizolam positives in this study were also positive for etizolam. All urine samples were treated in the same way with the same solutions therefore if pH changes or etizolam breaking down to metizolam in the instrument is occurring, why has this only been seen in some samples and not in others, which were also positive for etizolam. A study into the metabolism of metizolam found that less than one percent (0.3%) of the parent compound was found in urine unchanged but was still detectable up to 46 hours in urine. (Kintz *et al.*, 2017) With such a small percentage found in urine it seemed unlikely the metizolam detected in this study was from metizolam use. To investigate the source of metizolam sample DC 53 was re-extracted along with a further 7 urine samples from a previous study that were also positive for both etizolam and metizolam. These samples were ran on the method described in section 4.3.4 chapter 4 but in addition the theoretical metizolam metabolite MRMs described in Kintz *et al*, 2017 were monitored. (Kintz

et al., 2017) There was no certified reference standard available for this metabolite. It is expected that the LLE used was capable of extracting the theoretical metabolites as LLE is a broad extraction technique and is adequate for all the other drugs in the method however testing this using a certified reference standard is required to prove this. The parameters used to screen for the theoretical metizolam metabolite are displayed in Table 55. In addition to this experiment, etizolam in reconstitution solution at concentrations 1 mg/L and 10 mg/L were injected down the LC-MS/MS urine method in order to determine if etizolam is losing a methyl group due to the temperature in the ion source.

Table 55: Theoretical metizolam metabolite MRMs

Q1	Q3
345.058	327.043
345.058	291.038
345.058	276.013

According to Kintz *et al*, these transitions are of the main hydroxylated metabolite of metizolam.

The samples screened negative for the metizolam metabolite therefore it is likely the metizolam detected in these urine samples is from the metabolism or degradation of etizolam rather than consumption of metizolam. The results from the 10 mg/L etizolam injection are shown in Figure 53, the etizolam peak can be seen at the top panel, the middle and bottom panels are the metizolam QT and QL windows, the lack of response of metizolam excludes the possibility of in-source etizolam degradation.

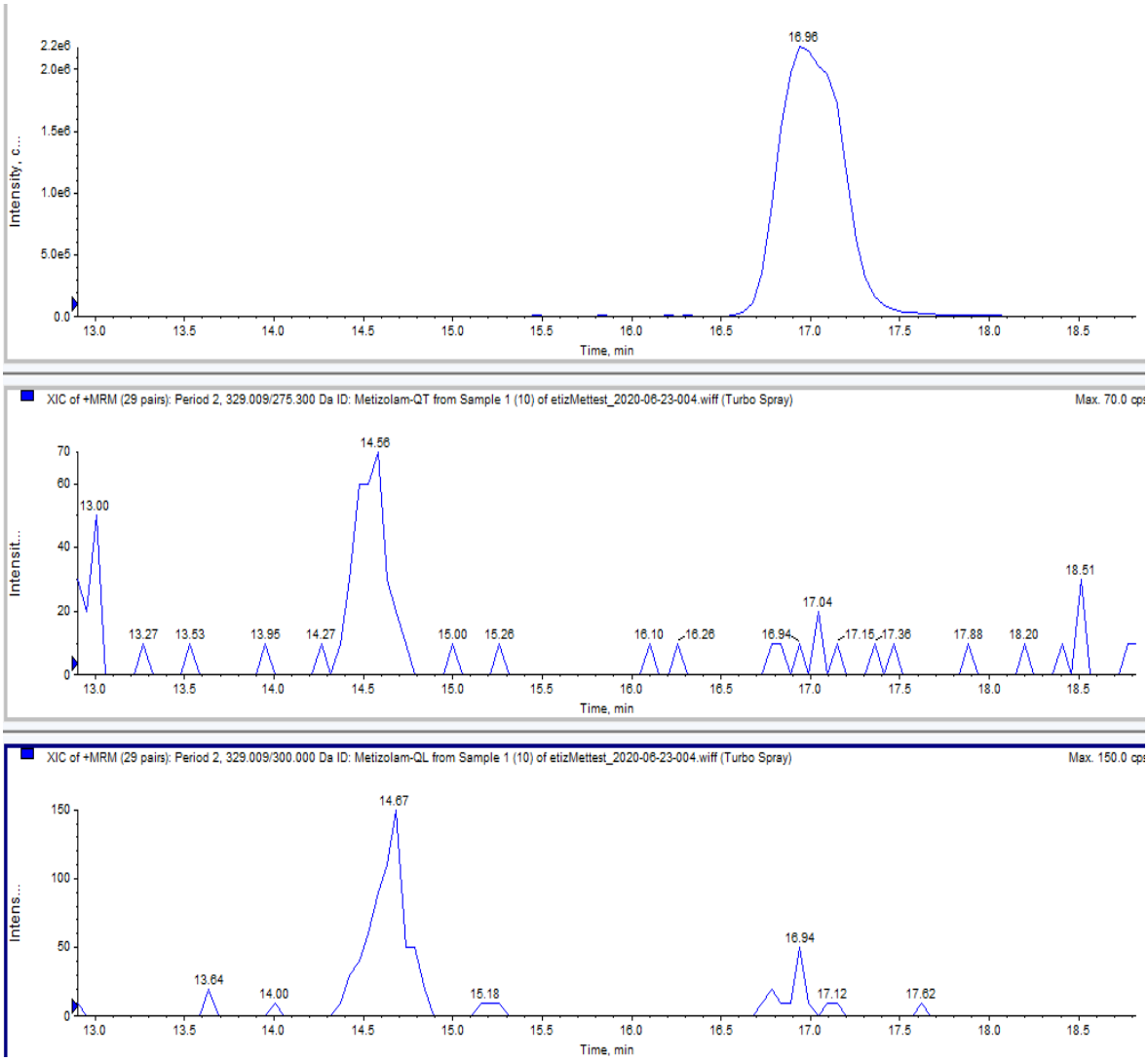

Figure 53: Chromatogram of a 10-mg/L etizolam injection and the lack of metizolam response

An article by Zawilska and Wojcieszak identified metizolam as a metabolite of etizolam (Zawilska and Wojcieszak, 2019) however give no further details. A Japanese study investigated the contributed of the cytochrome P450 isoforms in the *in vitro* metabolism of benzodiazepines including etizolam. (Niwa *et al.*, 2005) They concluded that CYP3A4 had the highest activity in etizolam metabolism however they also noted a minor contribution from CYP2C18 and an even lesser contribution from CYP2C19. Another study found that genetic polymorphisms of CYP2C19 could lead to variations in etizolam metabolism. (Fukasawa *et al.*, 2005) CYP3A4 is the predominant CYP in diazepam metabolism and is one of the enzymes responsible for the hydroxylation of diazepam to temazepam; (Niwa *et al.*, 2005) this is therefore the isoform responsible for the formation of alpha-hydroxyetizolam in etizolam metabolism. CYP2C18 was not detected in diazepam

metabolism however CYP2C19 was and is known to catalyse the demethylation to desmethyldiazepam (Niwa *et al.*, 2005) however this is best described as *N*-demethylation and therefore is not a mechanism in which metizolam can be produced from etizolam as the methyl group is absent from a carbon atom and not a nitrogen atom, see Figure 52 for the chemical structure comparison. By considering the metabolism of better-known benzodiazepines such as diazepam there does not appear to be a pathway that explains metizolam as a metabolite of etizolam. Another explanation for metizolam production is from the manufacture of etizolam possibly creating a metizolam by-product, this may explain why it is seen in some etizolam positive samples but not all as there are multiple ways to synthesise drugs. This is an unconfirmed theory due to the lack of literature on etizolam metabolism and metizolam in general.

5.5.2. Forensic Directorate psychiatric results

Urine samples were collected from 95 patients from January 2016 to November 2016 in NHS GGC FD facilities as part of the standard drug testing procedure.

5.5.2.1. Questionnaire responses

Each sample received had a corresponding form, which detailed the results of the immunoassay test, the drugs the patient was prescribed and the results of confirmation testing. Figure 54 shows the prescribed drugs that were mentioned on more than one occasion on the form submitted with each sample. Other drugs only mentioned once included amisulpride, clonazepam, quetiapine, propranolol, venlafaxine and haloperidol. Unlike the drug court cohort it was ensured that an individual did not donate twice to this study so the 95 samples were collected from 95 individuals. Only one form mentioned the prescribed use of benzodiazepines. This was the form with sample FP 73, it states the individual had 2 mg of diazepam approximately one week ago.

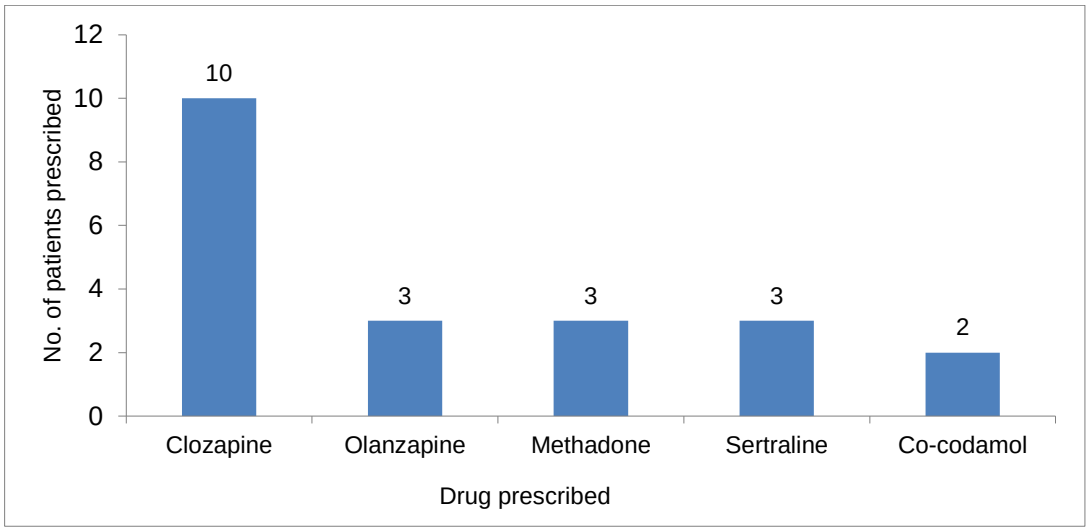

Figure 54: NHS GGC FD drugs mentioned on study forms

Table 56 displays the samples, which gave a positive immunoassay test. All other samples gave a negative response to all drug groups included in the immunoassay screen. Individuals being treated by NHS GCC FD are not given prior warning that they would be drug tested and the testing is done at random however they are aware that they are subject to random drug and alcohol testing.

Table 56: Screen results on the FD form submitted to FMS

Sample Number	Amphetamines	Benzodiazepines	Cocaine	Cannabis	Methadone	Opiates	Prescribed	Drug detected by GC/MS
13	N	N	N	N	P	N	Methadone	-
22	N	N	N	N	P	N	Methadone, olanzapine	-
49	N	N	N	N	N	P	Lansoprazole,aspirin, lamotrigine,clozapine	Codeine
50	N	N	N	N	N	P	None stated	Codeine and morphine
68	N	N	N	N	N	P	None stated	Codeine and morphine
74	N	N	N	N	N	P	None stated	Codeine and morphine
85	N	N	N	N	N	P	Co-codamol	Codeine and morphine
90	N	N	N	N	N	P	Co-codamol	Codeine and morphine
92	N	N	P	N	N	N	None stated	-

N = Negative response, P = Positive response.

5.5.2.2. Forensic Directorate psychiatric laboratory results

All urine samples were found to be negative for the compounds listed in Table 51, however oxazepam was detected at a concentration of <15 ng/mL (15 ng/mL cut-off was used) for FP073. According to the form, this individual had taken 2 mg of diazepam approximately one week ago however the initial urine immunoassay screening test had indicated a negative result. The immunoassay uses an oxazepam cut-off concentration of 200 ng/mL; (Abbott Architect, Benzodiazepine Manufacturer Kit Insert, 2018) so it is to be expected that this concentration would not have been detected. Due to the anonymised nature of the information provided it is not known if this patient was given this diazepam for a clinical reason or not or if they were in a low/medium or community setting. Consent was sought for every sample and therefore those likely to give a positive result may not have provided a sample. The apparent absence of benzodiazepines in this population is an

encouraging result as these drugs are not recommended in this group of individuals as mentioned in chapter 1 section 1.3, they may exacerbate symptoms and have a higher abuse potential than in some other populations.

5.6. Conclusion

This study found that diazepam was being commonly used in the sub-set of the SDC cohort tested but designer benzodiazepines were only detected in four samples. This gives a snapshot of the benzodiazepine use in these individuals during a six-month period however the voluntary requirement in this study could skew the results. There were more positive samples than noted on the questionnaires therefore proving the hypothesis correct for this population.

The forensic psychiatry (NHS GGC FD) results showed a population who do not appear to be using benzodiazepines; it is possible their treatment may be restricting their access to these drugs. It is thought that prescribing benzodiazepines to patients with a severe mental health diagnosis may exacerbate their condition and they are more at risk of abusing benzodiazepines than individuals who do not suffer from these conditions. Therefore it is unlikely that there would be many patients prescribed diazepam and other benzodiazepines. (Brunette *et al.*, 2003) There no positive samples therefore proving the hypothesis incorrect for this population.

5.6.1. Limitations

The individuals who took part in both studies gave consent as part of the ethical considerations. The author is not privy to how many individuals declined to be part of the study; it is possible those more likely to give a positive result did not consent to the additional testing. There was no reward offered to take part in this study. The individuals are required to attend the Drug Court by appointment and therefore it is possible that they were careful about their drug use in the couple of days prior to their appointment. With these considerations it is possible that the positives found in study are an underestimate of the benzodiazepine use in both populations. The patients in the forensic psychiatry cohort are tested for drugs and alcohol randomly with less prior warning however they are aware that they are subject to random testing. The immunoassay results for the SDC were not available for comparison and therefore no conclusion can be drawn on their use and level of success. It would be expected that the analytes detected in this study

would also give a positive results as traditional benzodiazepines immunoassays have demonstrated cross-reactivity with some of the designer benzodiazepines, see Chapter 2. Future studies in populations such as the SDC so should ensure access to immunoassay results in order to compare their results to the confirmatory results.

6. Scottish Prison Service samples

6.1. Introduction

The Scottish Prison Service (SPS) carries out the Addiction Prevalence Testing (APT) annually in all 15 Scottish Prisons. This involves all individuals being admitted to or liberated from one of these prisons in the designated testing month to undergo a urine drug test. Participation in the APT is voluntary. (Drug Misuse and Treatment in Scottish Prisons, 2019)

The analysis carried out is a point-of-care test, which includes a broad range of drug groups: cannabis, benzodiazepines, opiates, methadone, amphetamine(s), methamphetamine(s), barbiturates, cocaine, buprenorphine and some prisons included tramadol. This test is an immunoassay; the principle of immunoassay is explained in chapter 2. There is no available information on the brand of the test used.

The *Scottish Prison Study 2015* was carried out by all of the 15 prisons in Scotland, this was an anonymous survey, and all prisoners were invited to take part in. There was a response rate of 55%. 80% of the respondents were convicted, whilst the rest were untried at the time of the survey. 92% of respondents were male with the average age being 33 years old. In this survey, 43% admitted they had used illegal drugs while in prison and 24% stated they had used drugs in prison within the last month. Of those who used drugs within the last month 39% stated they used benzodiazepines.(Carnie and Broderick, 2015) The Scottish Prison Study 2015 and the SPS APT suggest that benzodiazepine use is an issue in custody settings and therefore designer benzodiazepines should be investigated in a prison setting.

6.2. Aim

The aim of this study was to determine the extent of benzodiazepine use including designer benzodiazepines in individuals being admitted to and liberated from SPS facilities during November 2015. Hypothesis: The individuals admitted to prison will give more positive results for benzodiazepines than those being liberated.

6.3. Ethical considerations

NHS Ethical Approval was sought and granted from the NHS West of Scotland Research Ethics Committee under reference WS/15/0207. See Appendix 11.9.

6.4. Method and materials

6.4.1. Materials

The materials used are detailed in section 4.3.1 of chapter 4.

6.4.2. Extraction method

Urine samples were extracted using the LLE extraction method detailed in section 4.5.1.3 of chapter 4.

6.4.3. Instrumentation and method

The extracts were analysed using the LC-MS/MS method detailed in section 4.3.4 of chapter 4. This method was employed as a qualitative method with cut-off concentrations. The cut-off used for this method was 15 ng/mL. Table 51 details the analytes included in the method, this is the same panel used in chapter 5.

6.4.4. Sample collection

Unpreserved urine samples were collected from 7 out of 15 SPS facilities; HMP Addiewell, HMP Barlinnie, HMP Cornton Vale, HMP Edinburgh, HMP Greenock, HMP Low Moss and HMP Perth as part of the APT procedure.

Individual consent was obtained before the remaining urine sample was sent to FMS by courier for additional testing regardless of a positive or negative SPS APT result. There were no personal details pertaining to the individual on the tube of the urine sample, the only detail was a sticker, which said either ‘Admission’ or ‘Liberation’.

6.4.5. Data handling and statistical analysis

Once results had been generated in the Analyst software, they were copied onto a Microsoft® Excel® (version 14.7.3) spreadsheet and batch checked by a second toxicologist before any calculations were performed. The results copied include the peak areas of the analyte and the internal standard, the calculated values of the calibration standards, samples and QCs in mg/L and the datapath information. Microsoft® Excel® was then used to generate the charts and descriptive statistics within the results section. The spreadsheets were kept on a secure drive to protect the data.

6.5. Results and discussion

725 urine samples were received from the seven prisons that took part. The SPS publish weekly figures showing the population of Scotland's prisons. (*Scottish Prison Service*) The Scottish Prison population on the last week of November 2015 (the APT collection month) for over 21 year olds was 7,187. Females made up 5% of the Scottish Prison population on that week. 725 samples represents around 10% of the Scottish prisoner population, however the samples received were prisoners in transit, either entering or leaving the prison system and not the general prison population. The number of admission and liberation samples from each prison is summarised in Table 57.

Table 57: Admission / Liberation breakdown for the prison urine samples tested in this study

Facility	No. Admission Samples	No. Liberation Samples	Total no. of samples
HMP Addiewell	69	34	109*
HMP Barlinnie	109	63	173*
HMP Cornton Vale	62	25	87
HMP Edinburgh	27	35	62
HMP Greenock	6	20	26
HMP Low Moss	19	40	60*
HMP Perth	138	70	208
Total	430	287	725*

*Six samples from HMP Addiewell and one each from HMP Barlinnie and HMP Low Moss were not labelled as admission or liberation and could not be identified as either.

6.5.1. SPS results vs this study results

SPS reported the most common drug group detected was cannabis followed by benzodiazepines in the admission samples. Buprenorphine was the most commonly detected drug in the liberation samples.(Addiction Prevalence Testing for Performance Measurement Purposes 2015-16 (November 2015) 2016) Table 58 shows the results of the SPS testing for each drug group. They tested 1,579 samples overall.

Table 58: Result for the urine samples (n=1,579) of SPS APT

Drug	% of positive samples	
	Admission	Liberation
Cannabis	52	8
Benzodiazepines	35	6
Opiates	25	8
Methadone	6	3
Amphetamine(s)	3	0
Methamphetamines	2	1
Barbiturates	0	0
Cocaine	15	0
Buprenorphine	7	12
Tramadol*	4	1

*Tramadol was tested in 7 out of the 15 prisons therefore does not give a true prevalence.

Table 58 displays the data across all 15 prisons; this is more prisons than tested for in this custody study therefore the results are not directly comparable. However, Figure 55 shows the results produced for the SPS APT for benzodiazepines for the 7 prisons included within the study presented in this chapter.

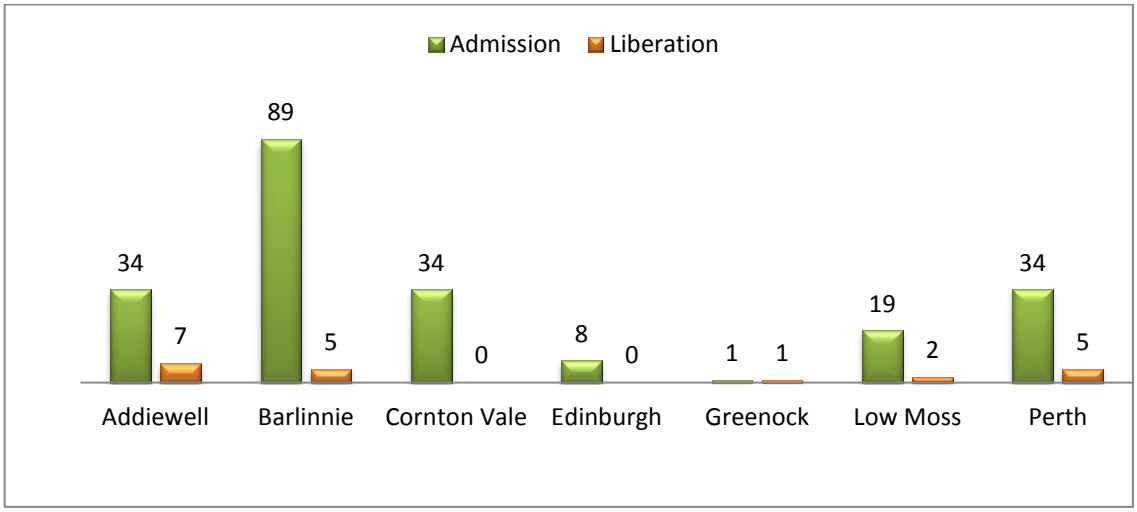

Figure 55: SPS APT results for benzodiazepines in the 7 prisons featured in this study.

The charts in Figures 56 and 57 show the number of positive results obtained by this study side-by-side with the SPS APT results. Only two prisons gave a higher incidence of positives in the SPS results, HMP Low Moss and HMP Barlinnie for admission samples. The liberation results in Figure 53 show that in all prisons this study found a higher incidence of positive results apart from HMP Addiewell, which

had the same number of positives. However the liberation samples overall gave a very low number of positives. It also should be noted that SPS tested a higher number of samples than this study, and not all samples collected were provided to FMS. SPS tested 986 samples from these 7 prisons, of these 725 were available for this study. These results suggest positive samples have been missed by the immunoassay testing kit used by the prisons and potentially underestimate how many have been missed as the SPS tested more samples than this study. Chapter 2 demonstrates how six benzodiazepines were successfully detected by the Immunalysis® Benzodiazepine ELISA kit however this was a laboratory based kit and not a point of care test. The test used by the prison was never detailed to the author therefore its cross-reactivity was not tested. This means it is not known if these samples have evaded detection due to cross-reactivity or the limit of detection. This further emphases the requirement for confirmation testing and why presumptive testing cannot be solely relied upon.

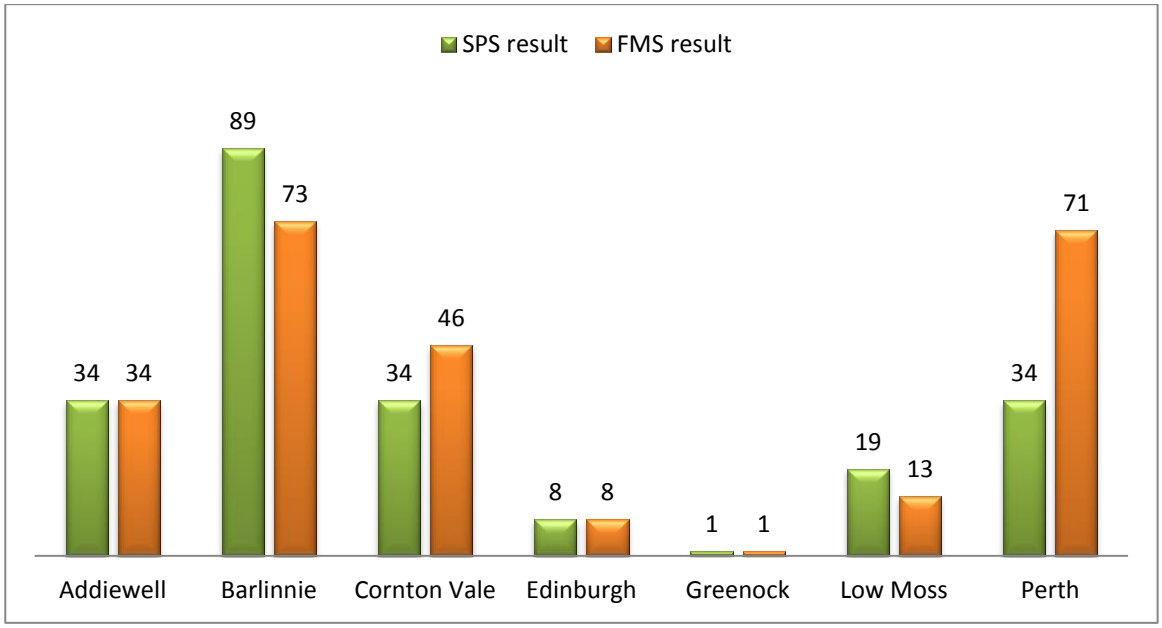

Figure 56: SPS APT results vs this prison study results in admission samples

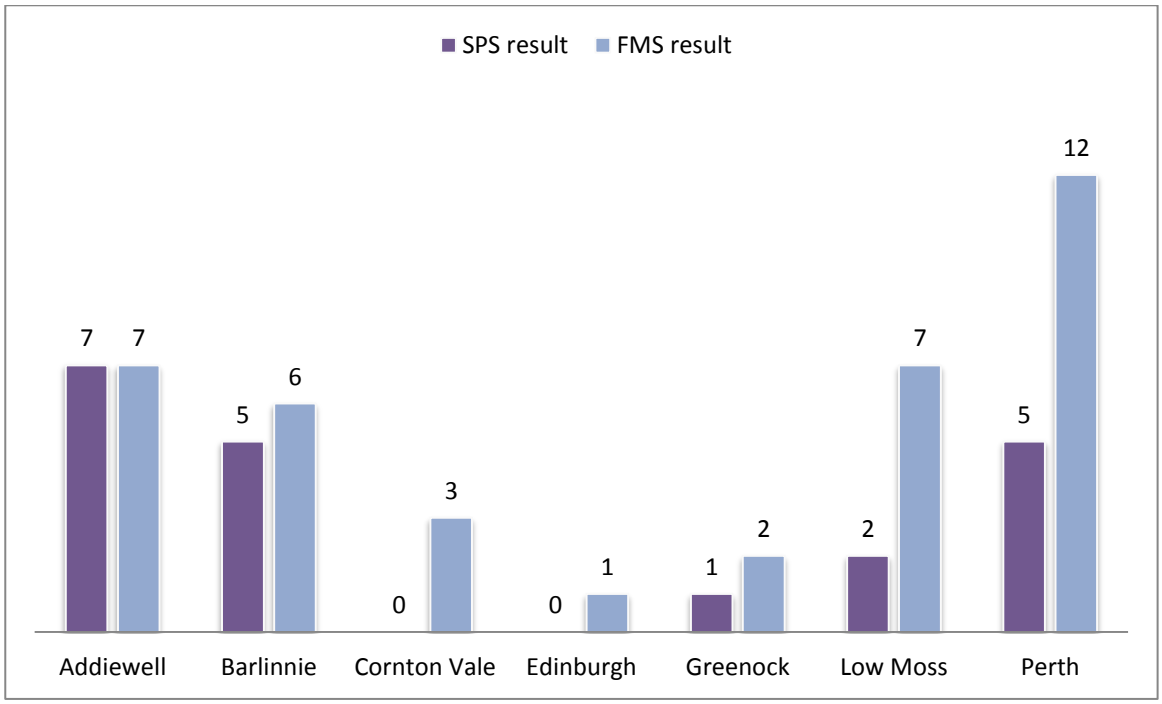

Figure 57: SPS APT results vs this prison study results in liberation samples

Table 59 shows the number of samples that tested positive for at least one benzodiazepine for admission, liberation and unlabelled samples for each prison. HMP Greenock which is a small prison designed to hold 249 prisoners only submitted 26 samples for this study. HMP Barlinnie, which is a larger facility and receives an average of 8,000 prisoner admissions each year sent 173 samples to this study. Interestingly HMP Cornton Vale, Scotland's female prison had the

highest percentage of positives for benzodiazepines overall.

Table 59: Number of positive urine samples for one or more benzodiazepine for this prison study

Facility	No. of positive samples	% of positive samples	Total no. of samples
HMP Addiewell	46	42%	109
HMP Barlinnie	80	46%	173
HMP Cornton Vale	49	56%	87
HMP Edinburgh	9	15%	62
HMP Greenock	3	12%	26
HMP Low Moss	20	33%	60
HMP Perth	83	40%	208
Total	290	40%	725

This study found that overall the 40% of the samples were positive for benzodiazepines, this is 5% more than the APT result however they did test 15 prisons compared to the 7 detailed here so it is not directly comparable.

6.5.2. Individual analyte results

There were six benzodiazepines that were negative in all samples in this dataset; these were alprazolam, deschloroetizolam, flubromazolam, clonazolam, meclonazepam and nifoxipam, however, no metabolites for these drugs were included in the analytical method used. Approximately 20% of alprazolam taken orally is excreted in urine unchanged so there is a detection window to some degree. (Fraser, Bryan and Isner, 1991) The nitrobenzodiazepines - clonazolam, meclonazolam and nifoxipam are likely to extensively metabolise and require the inclusion of metabolites in methods for urine detection. (Meyer *et al.*, 2016) Deschloroetizolam has found to be detectable in blood but not in urine and therefore also requires metabolites in urine analysis for successful detection. (El Balkhi *et al.*, 2017) Table 60 provides a summary of all positive findings and prevalence in the sample set.

Table 60: Summary of benzodiazepine urine positives for this prison study

	Oxazepam	Temazepam	Desmethyldiazepam	Diazepam	Lorazepam	Delorazepam	Lormetazepam	Etizolam	3OHPhenazepam	Metizolam	Phenazepam	Chlordiazepoxide	Nitrazepam	Pyrazolam	Flubromazepam	Diclazepam
HMP Addiewell	43	39	38	22	11	8	8	2	3	-	-	1	1	1	1	-
HMP Barlinnie	74	69	65	44	23	21	20	6	6	2	1	1	1	-	-	1
HMP Cornton Vale	49	45	41	28	8	7	5	3	1	-	1	2	1	-	-	-
HMP Edinburgh	9	8	6	5	-	-	-	-	1	-	-	-	-	-	-	-
HMP Greenock	3	3	3	1	-	-	-	-	-	-	-	-	-	-	-	-
HMP Low Moss	20	18	17	12	6	5	3	-	2	-	-	-	-	-	-	-
HMP Perth	83	76	72	41	11	3	5	11	8	5	4	1	-	-	-	-
Total no. of admission positives	237	225	210	139	54	41	39	20	18	7	5	5	3	1	1	1
Total no. of liberation positives	38	28	27	9	3	2	1	2	2	0	1	0	0	0	0	0
Total no. of unlabelled	6	5	5	5	2	1	1	0	1	0	0	0	0	0	0	0
Total Overall	281	258	242	153	59	44	41	22	21	7	6	5	3	1	1	1

6.5.3. Diazepam

Diazepam is the most prescribed benzodiazepine worldwide and metabolises to three other active drugs, desmethyldiazepam (nordiazepam) and temazepam, which both then metabolise to oxazepam. Desmethyldiazepam is also a metabolite of six other benzodiazepine drugs (chlordiazepoxide, clorazepate, halazepam, medazepam, prazepam and tetrazepam). (Ator and Griffiths, 1997)

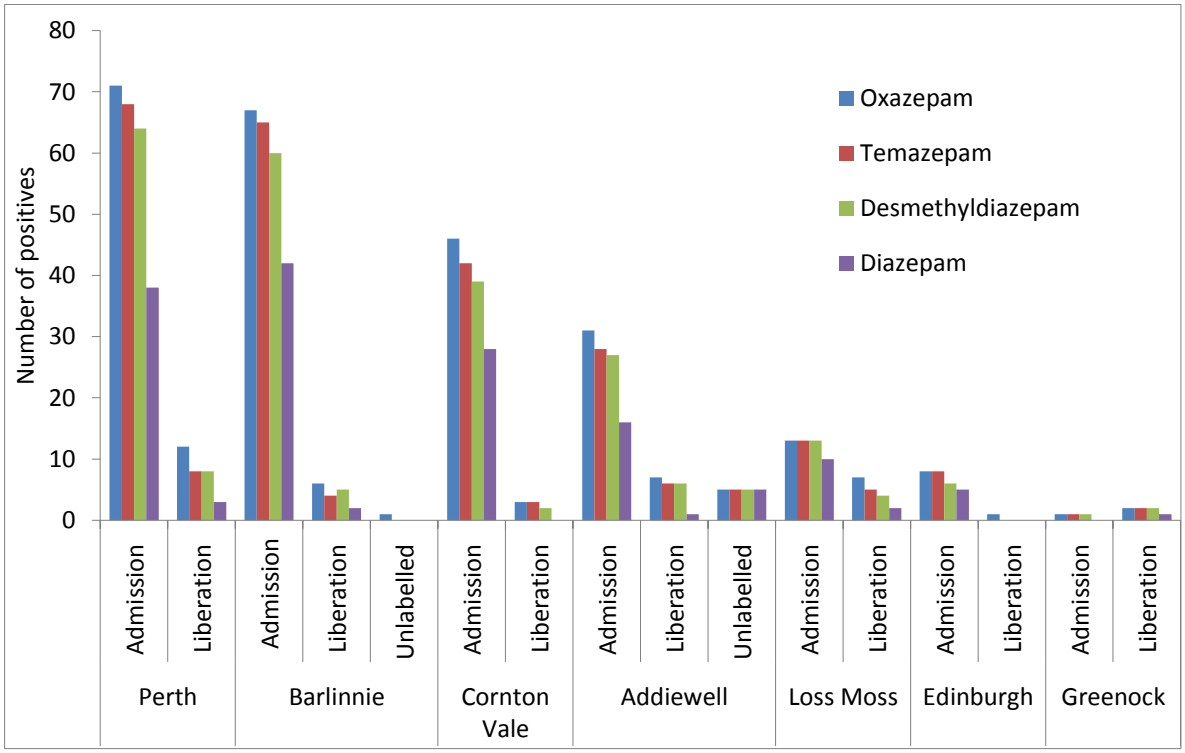

Figure 58: Diazepam and metabolite urine positive results for this prison study

Oxazepam was the analyte with the most number of positive samples in all prisons; this is unsurprising as it is the metabolite with the longest detection window from diazepam use. (Luk *et al.*, 2014; Lennestål *et al.*, 2008) It is likely the LC-MS/MS method cut-off of 15 ng/mL is lower than the cut-off used for the prison immunoassay, therefore more positives will be detected. Figure 58 shows the difference in the number of positives from admissions compared to liberations - this difference is more pronounced especially for HMP Perth, HMP Barlinnie and HMP Cornton Vale. Figure 59 shows the same data but displayed as a percentage positive, this allows the positivity rate between the prisons to be compared more easily. Figure 59 shows that HMP Cornton Vale has the highest rate of positivity for the diazepam metabolites. The graph in Figure 59 does not contain the samples which were not labelled. The one unlabelled sample from HMP Barlinnie

was positive for oxazepam and five out of the six HMP Addiewell unlabelled samples were positive for oxazepam, temazepam and desmethyldiazepam.

An oxazepam positive in urine indicates use of diazepam, temazepam or oxazepam. The oxazepam-only positive samples are likely to be from diazepam use, although use of other benzodiazepines cannot be excluded.

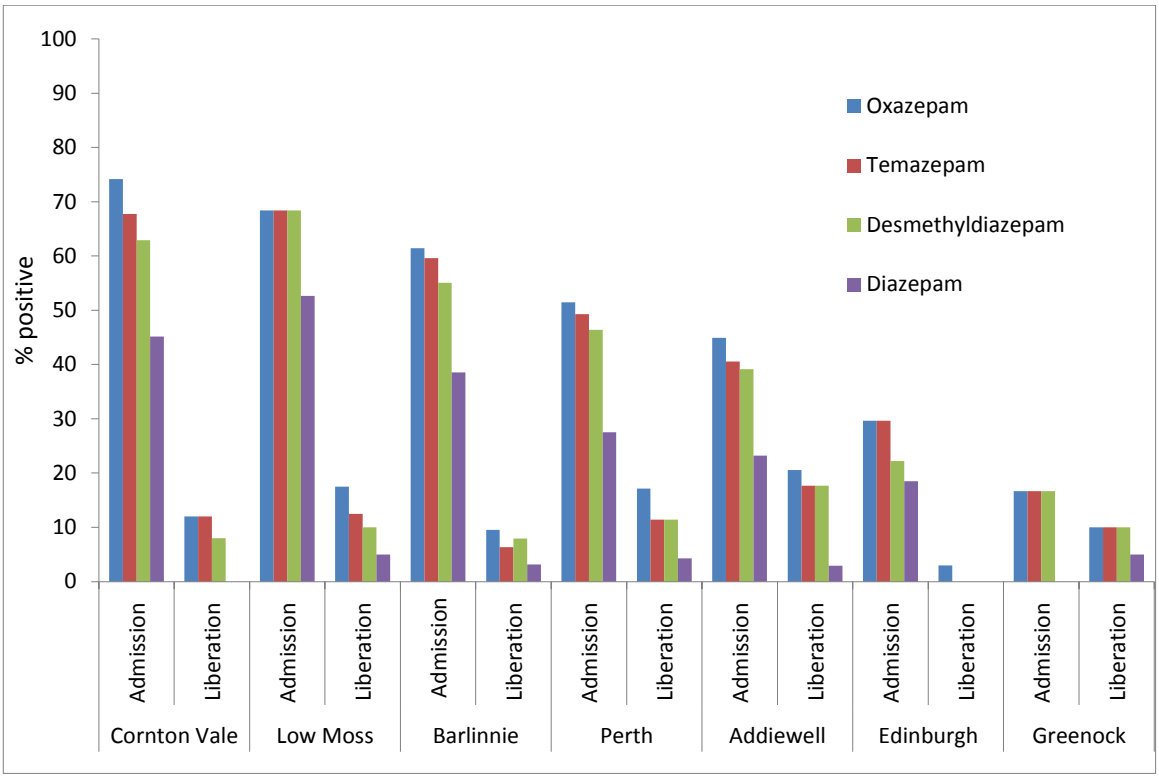

Figure 59: Diazepam and metabolite urine positive results for this study by percentage

Oxazepam was present without any other metabolites in four samples from HMP Addiwell, four samples from HMP Barlinnie, four samples from HMP Cornton Vale, one sample from HMP Edinburgh, two samples from HMP Low Moss and seven samples from HMP Perth. This equates to 8% of the oxazepam positive-samples with no other diazepam metabolites detected. Oxazepam is prescribed for the treatment of anxiety and insomnia, in Scotland but it is not common - in 2015/16 oxazepam was dispensed 8,936 times versus the 874,810 times diazepam was dispensed. (Information Services Division Scotland, 2016) One HMP Barlinnie sample was positive for oxazepam and desmethyldiazepam which is likely to show use of chlordiazepoxide due to the absence of temazepam.

It is reasonable to speculate that all of the positive results for oxazepam,

temazepam and desmethyldiazepam came from diazepam use. Many samples are positive for the diazepam metabolites but not diazepam itself, which is not unusual in urine samples. (Luk *et al.*, 2014)

6.5.4. Diclazepam

Diclazepam has been observed amongst Scottish fatalities in the past six years and in drug seizures. (Scotland, 2016) It is not always clear if users seek diclazepam specifically or they are unknowingly consuming it in ‘street Valium’ pills.(Scotland, 2016) Diclazepam metabolites were the most common analytes detected after the diazepam metabolites. Table 61 displays the number of diclazepam and metabolites positives. Diclazepam is metabolised to delorazepam and lormetazepam, which are then both metabolised to lorazepam, which is turned into a glucuronide for excretion, see Figure 11. One sample may be counted several times within Table 61 depending on how many metabolites were positive within that sample. Samples from HMP Edinburgh and HMP Greenock were all negative for diclazepam and metabolites and therefore Table 61 only shows the results of five prisons.

Table 61: Diclazepam and metabolites urine positive results for this prison study

Prison	Analyte	No. of admission positives	No. of liberation positives	No. of unlabelled positives	Total No. of positives
HMP Addiewell	Lorazepam	9	0	2	11
	Delorazepam	7	0	1	8
	Lormetazepam	7	0	1	8
	Diclazepam	0	0	0	0
HMP Barlinnie	Lorazepam	23	0	0	23
	Delorazepam	21	0	0	21
	Lormetazepam	19	1	0	20
	Diclazepam	1	0	0	1
HMP Cornton Vale	Lorazepam	8	0	0	8
	Delorazepam	7	0	0	7
	Lormetazepam	5	0	0	5
	Diclazepam	0	0	0	0
HMP Low Moss	Lorazepam	4	2	0	6
	Delorazepam	3	2	0	5
	Lormetazepam	3	0	0	3
	Diclazepam	0	0	0	0
HMP Perth	Lorazepam	10	1	0	11
	Delorazepam	3	0	0	3
	Lormetazepam	5	0	0	5
	Diclazepam	0	0	0	0

Lorazepam was detected without any other diclazepam metabolites in some samples (n =1 - HMP Low Moss, n = 1 - HMP Cornton Vale, n = 2 - HMP Barlinnie, n = 3 - HMP Addiewell, n = 8 - HMP Perth). This equates to 25% of lorazepam positive-samples with no other diclazepam metabolites detected. Lormetazepam was detected on its own in one liberation sample from HMP Barlinnie and one admission sample from HMP Perth. All positive samples for diclazepam and its metabolites were in admission samples with the exception of two unlabelled samples from HMP Addiewell, one sample previously mentioned from HMP Barlinnie, one sample from HMP Perth and two from HMP Low Moss. Admission samples have a higher positivity rate just as the diazepam results showed.

Lorazepam-only positives can be a result of diclazepam, lormetazepam or lorazepam use. Both lorazepam (used in the treatment of psychosis) and lormetazepam (used in the treatment of insomnia) are prescribed in Scotland. Lorazepam is not commonly prescribed and was dispensed 97,857 times in 2015/16 year period compared to the 874,810 times diazepam was dispensed.(Information Services Division Scotland, 2016) Lormetazepam is even less common and was dispensed 3,541 times in that one-year period. Delorazepam (also a drug in its own right) is a metabolite of both diclazepam and cloxazolam. It is not licensed for use in the UK and is not included in the British National Formulary (BNF).(British Medical Association) Cloxazolam is also not prescribed in the UK. Delorazepam was always detected along with other diclazepam metabolites in this study. This makes delorazepam the most suitable metabolite for targeting diclazepam use in urine sample; it is less likely to have come from another source like lorazepam.

6.5.5. Other designer benzodiazepines

Etizolam was the second most common designer benzodiazepine (following diclazepam) found in this dataset with 22 positives detected overall (7.6% of the positive samples, 3% overall). Figure 60 displays the number of other designer benzodiazepine urine positives excluding diclazepam. Figure 61 displays the designer benzodiazepine urine positive excluding diclazepam broken down by prison. HMP Perth had the most etizolam positives at 11; they were all found in admission samples. HMP Barlinnie had two liberation samples that were positive for etizolam (3% of HMP Barlinnie liberation samples) one of these samples was also positive for 3-hydroxyphenazepam. 3-hydroxyphenazepam is also a drug not just a metabolite therefore its presence may indicate 3-hydroxyphenazepam consumption and not phenazepam use, the six phenazepam positive samples were all also positive for 3-hydroxyphenazepam. One study found phenazepam was detected in samples fortified with 3-hydroxyphenazepam only, likely due to thermal instability (Crichton *et al.*, 2015) therefore determining the source of this, from phenazepam or 3-hydroxyphenazepam consumption is particularly difficult. The samples in this study were collected in November 2015 and 3-hydroxyphenazepam was reported to the EMCDDA in 2016. (EMCDDA., 2015) This suggests phenazepam metabolism is the more likely explanation for 3-hydroxyphenazepam detection however consumption is cannot be ruled out.

Metizolam was positive in seven admission samples overall. All metizolam positive samples were also positive for etizolam. As previously described in Chapter 5, Section 5.5.1.3 metizolam positives could be the result of etizolam use rather than metizolam itself.

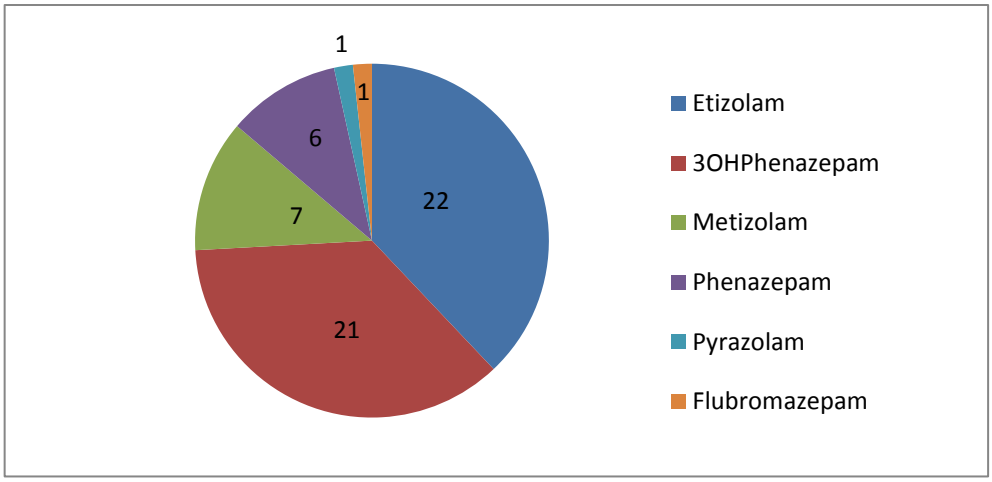

Figure 60: Designer benzodiazepine prison urine sample positives-excluding diclazepam

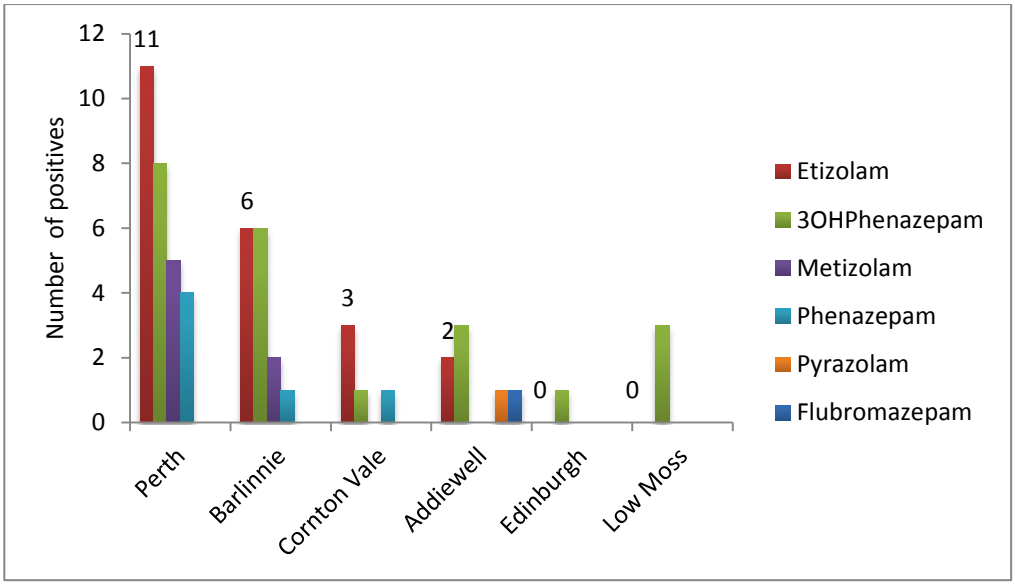

Figure 61: Designer benzodiazepine urine sample positives by prison-excluding diclazepam

6.5.6. Poly-drug use

Figure 62 shows the number of benzodiazepines present in positive samples. The majority (73%) of positive samples had one drug present. This chart has been made with the assumption that oxazepam, temazepam and desmethyldiazepam have come from diazepam use, and therefore one drug has been consumed. It was also assumed that samples positive for delorazepam, lorazepam and lormetazepam were from diclazepam consumption. A sample positive for both etizolam and metizolam has been counted as consumption of etizolam only, and 3-hydroxyphenazepam and phenazepam together has been counted as consumption of phenazepam only. It is therefore possible that the values used to create this chart are an underestimate.

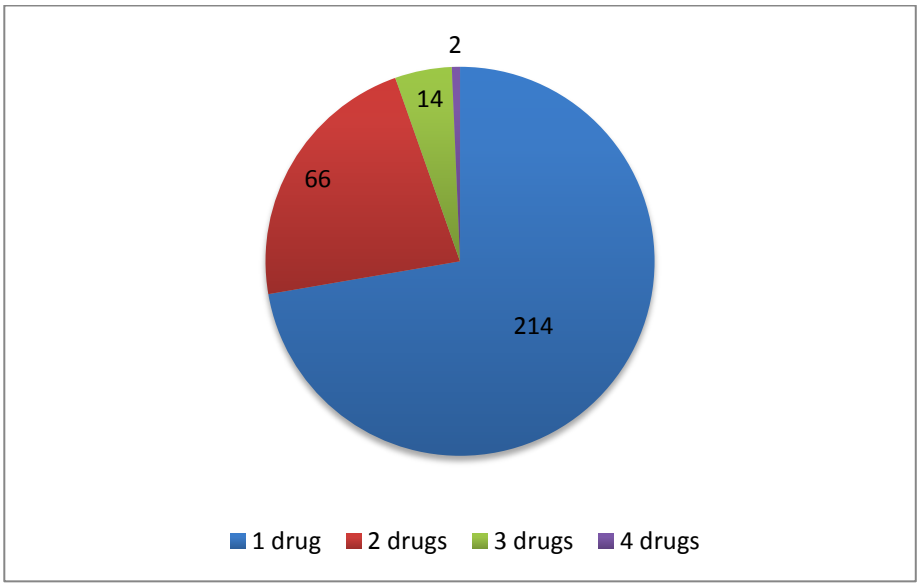

Figure 62: Poly-Benzodiazepine use within positive prison urine samples

6.6. Conclusion

Overall this data shows that the majority (60%) of participants in this study were negative for all benzodiazepines tested. Diazepam metabolites were the most commonly detected for all prisons; particularly in admission samples. It is not possible to know if these positives were the result of prescribed diazepam use, due to the anonymised nature of the study. Diclazepam use was mostly detected amongst admission samples; HMP Barlinnie had the highest number of diclazepam metabolite positives with 21% of admission samples. Other designer benzodiazepines did not have as high a presence overall, at 3% for etizolam, the

second most common designer benzodiazepine. The vast majority (73%) of positive samples for designer benzodiazepines showed use of one benzodiazepine drug.

Benzodiazepine use is occurring in the Scottish prison system. This data shows that the admission samples have a greater incidence of positives than the liberation samples. It suggests that individuals are going into prison whilst using benzodiazepines but are likely to come out of prison having abstained from benzodiazepine use. This may indicate that benzodiazepines are difficult to obtain in prison but it should also be noted that more admission samples were tested and consent was required to be included in this study.

6.6.1. Limitations

Due to the need to obtain consent for this study, this may not be a true representation of the use of benzodiazepines within the prison population. There is the possibility of collection bias and that those who knew they had been taken drugs may have refused to participate. This also only gives data on individuals entering or leaving the prisons so therefore cannot show benzodiazepine use amongst the general prison population. It would also be useful to know if the individuals providing the samples were prescribed any benzodiazepines in order to determine if the results are truly illicit use of a drug. Random urine sampling for drugs would give a more comprehensive overview of the illicit drugs being consumed while in prison.

7. Designer benzodiazepines in post-mortem blood samples

7.1. Introduction

There are few articles within the scientific literature that have explored the concentrations of designer benzodiazepines in human tissue (Høiseth, Tuv and Karinen, 2016; Moosmann *et al.*, 2013a; Moosmann *et al.*, 2013b; Moosmann, Bisel and Auwärter, 2014; Crichton *et al.*, 2015) and even fewer that have considered these drugs in post-mortem samples. (Shearer *et al.*, 2015; Lehmann *et al.*, 2019; Heide *et al.*, 2020; Crichton *et al.*, 2015; Mei *et al.*, 2019) Diclazepam and pyrazolam post-mortem blood concentrations are discussed in one study for a single case. (Lehmann *et al.*, 2019) The most comparable study to this one in the literature is a Norwegian article that examined designer benzodiazepine in living offenders as well as in post-mortem blood. (Heide *et al.*, 2020) This article revealed diclazepam to be the most commonly detected designer benzodiazepine in both populations. In the post-mortem data diclazepam was detected 13 times in 6,500 and etizolam was only detected twice. (Heide *et al.*, 2020) The study has the advantage of a large dataset however the rate of positive cases in the post-mortem blood is low and is much lower than in Scotland, however the study does not include any metabolites in their method which would potentially increase their positivity rate. They include lorazepam analysis but this was detected as part of their routine investigation as its own drug and not included because it is a diclazepam metabolite. There was also the disadvantage of no causes of deaths included within the study. These PM studies have been summarised in section 1.10 of chapter 1. The DRD data discussed in section 1.9.1 of chapter 1 shows how common etizolams presence is in Scotland, however this is not the only illicit benzodiazepine in circulation over the last few years. Diclazepam, pyrazolam and flubromazepam were reported to the EMCDDA not long after etizolam and therefore became drugs of interest in this study. It is important to understand if the individuals in Scotland who are dying are taking these drugs, in DRDs as well as other non-drug related deaths. Collating this data will provide concentration ranges, which can be consulted by toxicologists and pathologists to aid their interpretations.

7.1.1. Post-mortem toxicology in Scotland

Scotland is split into three Scottish Fatalities Investigation Units (SFIU) named SFIU North, SFIU East and SFIU West. Figure 63 shows the geographical area each unit covers. The East and West area serves around 3 million out of the 5.4 million Scottish population. (Shearer *et al.*, 2015)

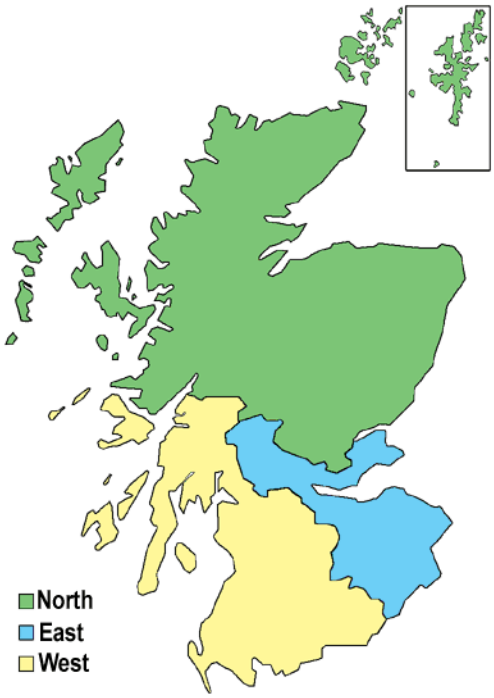

Figure 63: The areas of Scotland divided by the three Scottish Fatalities Investigation Units.
(reproduced with permission under the Open Government License
http://www.nationalarchives.gov.uk/doc/open-government-licence/version/3/)

The Forensic Toxicology Service (FTS), which is part of Forensic Medicine and Science (FMS) at the University of Glasgow, carries out the post-mortem toxicology testing for SFIU West and SFIU East and the Tayside area of SFIU North. Since 2015 the FTS has analysed more than 3,000 cases for post-mortem toxicology per year, this is approximately 90% of the Scottish post-mortem toxicology casework.

7.1.2. Aim

The aim of this study was to review all post-mortem casework positive for the designer benzodiazepines, diclazepam and two of its metabolites (delorazepam and lormetazepam), flubromazepam and pyrazolam from 2014 to 2018. Data collated included drug concentrations, other drugs present, the circumstances surrounding the death and the recorded cause of death. This information will give toxicologists a clearer idea of how to interpret the concentrations encountered in post-mortem casework. This, in turn could assist pathologists in understanding if the drug has contributed to the cause of death or not.

7.1.3. Ethical considerations

Ethical approval was sought and granted from the University of Glasgow Medical, Veterinary and Life Sciences Ethics Committee under reference 200180192**.** (See Appendix 11.10).

7.2. Method

7.2.1. Method and extraction

Blood samples were extracted using the SPE extraction method detailed in Section 3.3.5 of chapter 3 as part of the post-mortem toxicological investigation. The instrumentation and analytical method used in this study is described in section 3.3.4 in chapter 3. This designer benzodiazepine method included diclazepam and two of its metabolites (delorazepam and lormetazepam), flubromazepam and pyrazolam. These were the designer benzodiazepines that directly followed etizolam and had been reported to the EMCDDA when the method was created.

7.2.2. Data Collection

An in-house database containing information on all cases submitted for analysis to FTS was used to identify the designer benzodiazepines positive cases between January 2013 and December 2018. This gave an approximate six-year time period. Due to the number and variety of cases received during this time period, not every case was analysed for the designer benzodiazepines. On receipt, all cases are reviewed and case circumstances, such as items found at the scene or statements from witnesses, and the volume of sample available were used to decide if testing was appropriate. Therefore the number of positive cases found is

likely to be an underestimation of the true prevalence in the casework received by FTS. Phenazepam has been tested for since 2012 and etizolam from 2013 for cases received to FTS, these will not be detailed in this PM study. Diclazepam, flubromazepam and pyrazolam were tested for in cases deemed appropriate by the pathologist or the toxicologist since 2014. From September 2018 all drug related deaths were tested for designer benzodiazepines. The results of the routine benzodiazepine analysis prompted the analysis for the designer benzodiazepines in some cases for example a positive lorazepam may indicate a diclazepam positive case. Every effort was made to ensure cases were not missed for designer benzodiazepine analysis but it is still a possibility.

The positive cases identified were examined to extract the following information:

1. *Basic demographics.* This included the age, sex and SFIU region.

2. *Toxicological findings.* This included the post-mortem femoral blood concentrations of diclazepam and metabolites (delorazepam and lormetazepam), flubromazepam and pyrazolam as well as the presence of other drugs.

3. *Cause of death.* This is the cause of death including contributory factors and the categories of death (e.g drug-related, unascertained).

7.2.3. Data handling and statistical analysis

Once results had been generated by the in-house database, they were copied onto a Microsoft® Excel® (version 14.7.3) spreadsheet and checked by a second toxicologist before any calculations were performed. Microsoft® Excel® was then used to generate the descriptive statistics where necessary in addition to the percentiles for the diclazepam blood concentration data and to conduct a paired two sample t-test to determine if there was a statistical difference between the concentration included or excluded in the cause of death for pyrazolam and flubromazepam positive cases. The spreadsheets were saved on a secure drive to protect the data.

7.3. Results and discussion

Over the six-year period examined, 1691 cases were analysed using the SPE, LC-MS/MS method (see section 3.3.4 of chapter 3) including diclazepam and its two metabolites, flubromazepam and pyrazolam. There was a total of 369 cases positive for the designer benzodiazepines tested in blood (22% overall), 354 of

these were diclazepam positive (21% overall). A case positive for delorazepam is counted as a diclazepam positive as delorazepam is the major metabolite, there were 126 instances of delorazepam positive cases which were negative for diclazepam and lormetazepam. These cases may have also been positive for lorazepam, which was detected in a separate analytical method.

The 369 total positive cases included 18 flubromazepam positive cases (1% of all cases analysed), 7 of which were also diclazepam positive cases and four were positive for pyrazolam. The 369 total positive cases also included 9 pyrazolam positive cases (0.5% of all cases analysed), of which 1 was also diclazepam positive.

7.3.1. Diclazepam results

Figure 64 shows the number of cases positive for diclazepam and diclazepam metabolites in post-mortem blood from 2013 to 2018. Diclazepam positives peaked in the year 2016, they were positive in 49% of post-mortem cases analysed for designer benzodiazepines. Table 62 shows the percentage of diclazepam positive cases in the FTS post-mortem casework from 2013 to 2018. There was a dramatic dip in diclazepam positives in 2017 despite an increase in the number of cases analysed. This appears to be the direct impact of the Psychoactive Substances Act 2016, which came into force in May 2016. Interestingly, a 2019 study that monitored self-reporting of NPS use in the admissions to a London hospital found that there was no decrease after the Act came into force. (Webb *et al.*, 2019) However, drugs like synthetic cannabinoids and cathinones are popular in admissions to this emergency department. (Webb *et al.*, 2019)

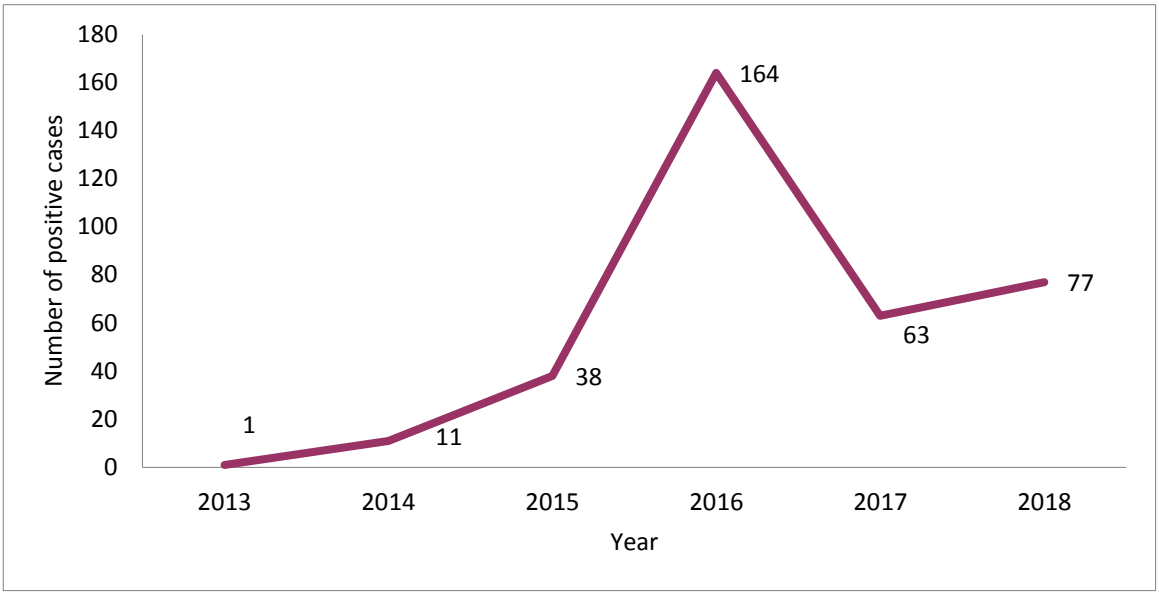

Figure 64: Diclazepam positive cases in FTS post-mortem blood casework casework over a six-year period (2013-2018)

Table 62: Percentage of diclazepam positive cases in FTS post-mortem casework

Year	Number of post-mortem toxicology cases	Number of cases analysed for designer benzodiazepines	% of analysed cases positive
2013	2563	1	100
2014	2991	50	22
2015	3181	64	59
2016	3367	337	49
2017	3407	413	15
2018	3703	826	9

7.3.1.1. Demographics

This dataset found there were more males positive for diclazepam in post-mortem cases than females. The difference in male to female positive cases was particularly noticeable in 2016; when there was more than double the number of males compared to females, see Figure 65. The “Drug-related deaths in Scotland in 2018” report produced by the National Records of Scotland found that males made up 72% of drug related deaths. (National Records of Scotland, 2019)

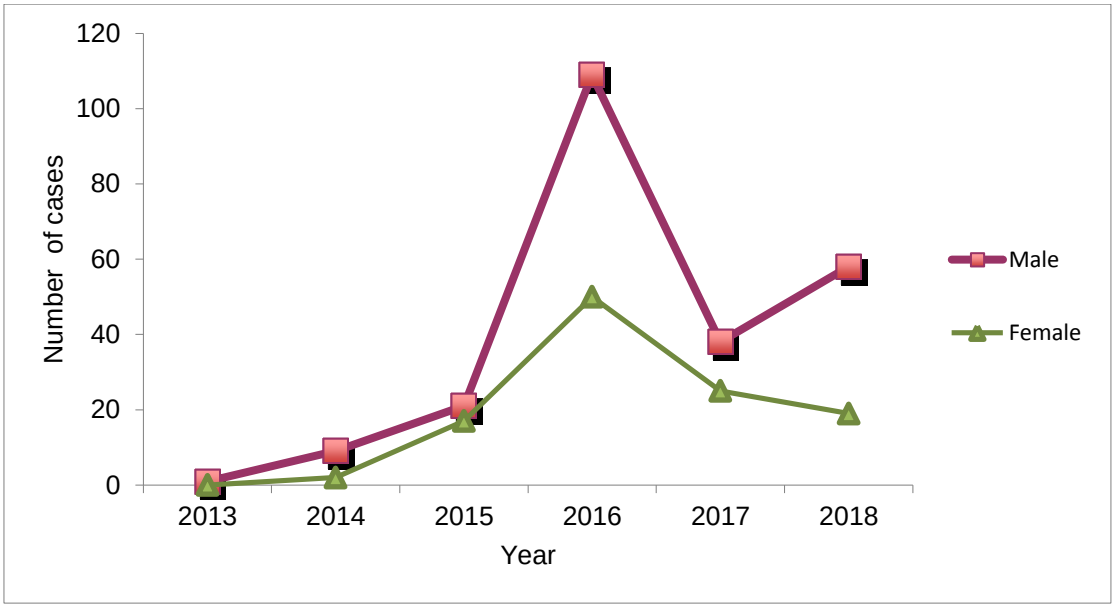

Figure 65: Sex of diclazepam positive cases in FTS post-mortem casework over a 6-year period (2013-2018)

The mean age found in this study remained consistent for the cases from 2014 to 2018, (range, 18-75 years old). The median age was 41 years old for both male and female. The number of cases positive for diclazepam in each age range is presented in Figure 66. There was only one case from December 2013 and this individual was 22 years old.

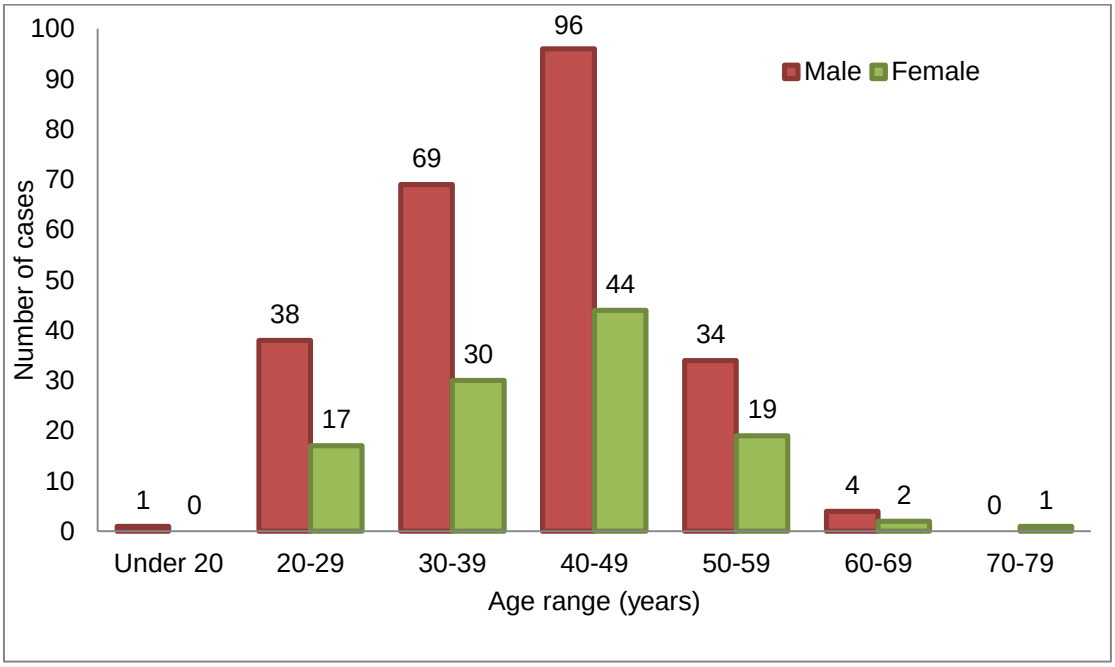

Figure 66:The range of ages for male and female in diclazepam positive post-mortem cases cases over a 5-year period (2014-2018)

The “National Records of Scotland, Drug-related deaths in Scotland in 2018” report found that the two age groups encompassing the 25 to 44 year olds had the largest number of DRDs. Figure 67 shows the rates for each age group over an 18-year period. (National Records of Scotland, 2019)

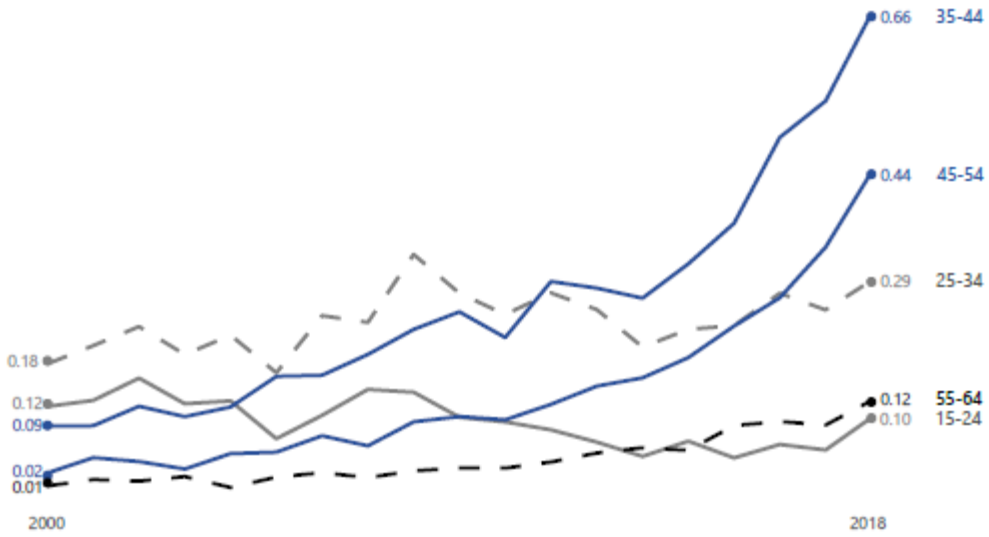

Figure 67: Drug-related death rates rates over an 18-year period (2000-2018) by age group

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7.3.1.2. Toxicological findings

Figure 68 displays the range of concentrations detected, and shows that diclazepam is more likely to be detected at the lower end of the concentration range. The mean and median concentrations of the diclazepam positive cases are displayed in Table 63. The LLOQ for this method was 0.005 mg/L; a positive sample was one that gave a peak area ratio of 50% or above the peak area ratio of the LLOQ. The ULOQ was 0.20 mg/L, for most cases sample dilution was carried out to give a quantitative value if required, however >0.20 is used for the purposes of this chart.

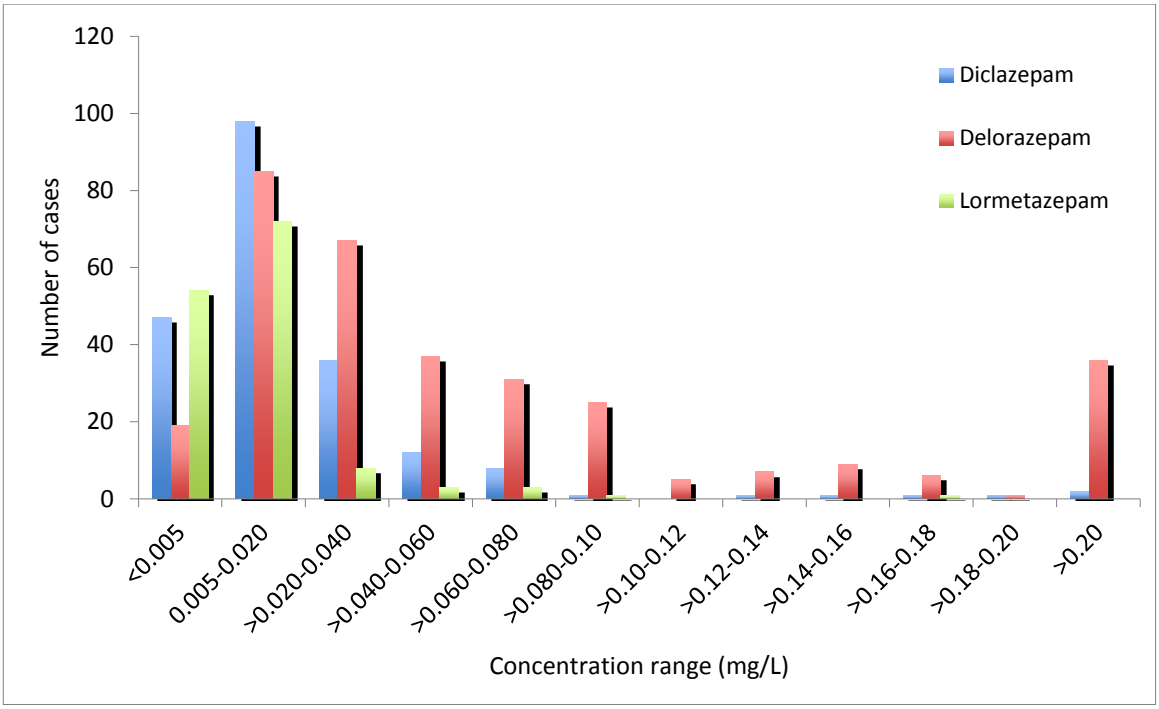

Figure 68: The range of diclazepam and metabolite post-mortem femoral blood concentrations detected over a 6-year period (2013-2018)

Lormetazepam is also likely to be detected at the lower end of concentration range, (range, 0.005 – 0.020 mg/L for all three analytes). Lormetazepam is an active metabolite that is then further metabolised to lorazepam, which is also active; see section 1.7 of chapter 1. Delorazepam is the active metabolite that is most likely to be detected over a wider range of concentrations according to this dataset. A study by Moosmann *et al* (Moosmann, Bisel and Auwärter, 2014) demonstrated that diclazepam has an elimination half-life of around 42 hours and the metabolites delorazepam and lormetazepam could be detected in urine for up to 6 days and 11 days, respectively. This study also carried out a self-administration experiment; a 43 year old ingested a 1 mg diclazepam tablet. The study found that the peak serum concentration for diclazepam was 0.003 mg/L and was reached after 3 hours, delorazepam was 0.002 mg/L and was reached after 36 hours, lormetazepam was 0.0003 mg/L and was reached after 6 hours. (Moosmann, Bisel and Auwärter, 2014) These concentrations are low compared to the average found in the post-mortem blood in this study however this is a single dose of 1 mg compared to potentially heavy consumption of the drug.

Table 63: Diclazepam and metabolites femoral blood concentration range and median in post-mortem cases over a 6-year period (2013-2018).

Analyte	Minimum (mg/L)	Lower quartilte (mg/L)	Median (mg/L)	Upper quartile (mg/L)	Maximum (mg/L)
Diclazepam (n=212)	0.005	0.011	0.017	0.033	0.400
Delorazepam (n=339)	0.005	0.020	0.043	0.098	1.500
Lormetazepam (n=144)	0.004	0.006	0.010	0.019	0.180

The study by Høiseth *et al,* which described designer benzodiazepine concentrations in living offenders of various crimes, found an 18-year-old male with a diclazepam blood concentration of 0.057 mg/L who was described to be "considerably impaired." There were no other drugs detected in this individual's blood. This was the highest concentration of diclazepam found in that study of 15 positive cases; the median concentration was 0.013 mg/L.(Høiseth, Tuv and Karinen, 2016) This is close to the median concentration for diclazepam in this study (0.017 mg/L).

A study from Australia (Partridge *et al.*, 2018), described a 23-year-old male who was found dead at home, he had a history of illicit drug use and had a post-mortem blood concentration of 0.07 mg/L for diclazepam and 0.01 mg/L for flubromazepam in addition to the synthetic opioid U-47700, the hallucinogen 2,5–dimethoxy-4-chloroamphetamine (also known as DOC), methyl amphetamine, amphetamine, lorazepam (not stated if the authors considered this a diclazepam metabolite or from additional use of lorazepam) and etizolam. The cause of death was given as "aspiration (of gastric contents into airways and lungs) due to mixed drug toxicity." (Partridge *et al.*, 2018)

Only eight cases in this study gave a diclazepam concentration in the range >0.06 – 0.08 mg/L (see Figure 68), therefore 0.07 mg/L is considered a moderately high diclazepam concentration in the context of this study.

Heide *et al* detected 13 diclazepam cases in post-mortem blood that had a median concentration of 0.0032 mg/L, range 0.0018 – 0.032 mg/L. This median is below the LLOQ of this study. The results of the Heide *et al* study are towards the low end of the concentration range used in this study, perhaps a more sensitive method would have detected more positives in this study however this study compensates by analysing for two metabolites which gives further evidence of

use. It is unfortunate Heide *et al* only gave diclazepam data as comparison of the metabolites, in particular delorazepam would have been particularly interesting as this has a much higher mean and median concentration than diclazepam.

The routine toxicological analysis of the post-mortem case samples identified additional drugs in the samples positive for diclazepam. The most commonly found drugs are displayed in Figure 69; the antidepressants category in the chart represents amitriptyline, mirtazapine, sertraline, paroxetine, venlafaxine and citalopram. The antipsychotics category represents quetiapine and olanzapine. The 6-monoacetylmorphine (6-MAM) positive cases in the graph include post-mortem blood and/or urine positives. The other drugs are all present in the post-mortem blood. The author did not carry out the toxicological analysis for these additional drugs; the hard work of the FMS technicians must be credited for this.

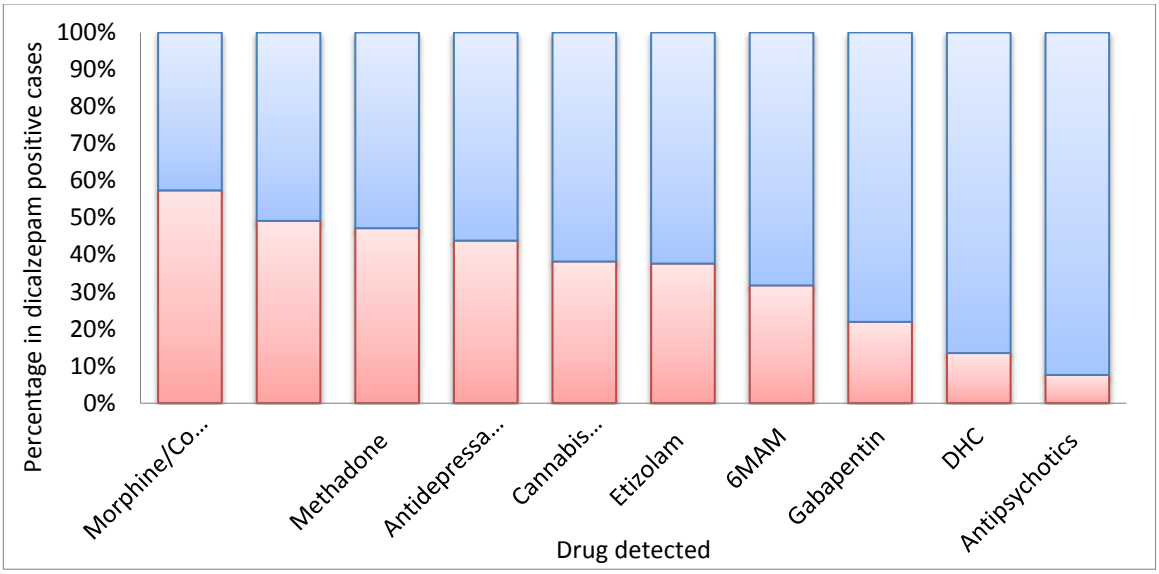

Figure 69: Other drugs found in diclazepam positive post-mortem cases over a 6-year period (2013-2018)

Figure 69 shows the most commonly found drugs in the diclazepam-positive samples were morphine and/or codeine. Morphine and/or codeine was found in 58% of the diclazepam positive cases. The concurrent use of opioids and benzodiazepines prolong the high from the opioids making benzodiazepines an attractive drug for heroin users. (EMCDDA., 2015) The detection of morphine and codeine in post-mortem blood samples can be from codeine use (as morphine is a metabolite of codeine), morphine and codeine use or heroin use. The morphine and codeine ratios were not determined. The presence of 6-MAM can assist with interpretation as it confirms heroin use. (Baselt) One hundred and twenty

diclazepam positive cases also tested positive for 6-MAM in either the post-mortem blood or urine confirming heroin use, another 59 cases were potentially heroin positive cases – 25 of these cases were positive for morphine and codeine with the remaining 34 cases positive for morphine but negative for codeine. There were 10 cases, which were positive for codeine only. DHC in the graph stands for dihydrocodeine, a prescription opioid. Other benzodiazepines, diazepam and its metabolite (DMD) and etizolam were also commonly detected. It was not always known if the user was intentionally taking diclazepam or if they were under the impression they were consuming diazepam or etizolam. Drug testing services such as the Welsh Emerging Drugs and Identification of Novel Substance (WEDINOS) (https://www.wedinos.org, 2016) have shown that users have purchased street diazepam or etizolam and have in fact been sold diclazepam. WEDINOS is a harm reduction service, which analyses substances submitted to them, this is helpful in giving in current market intelligence. Figure 70 is an example from the WEDINOS website showing a tablet submission they received in 2016.

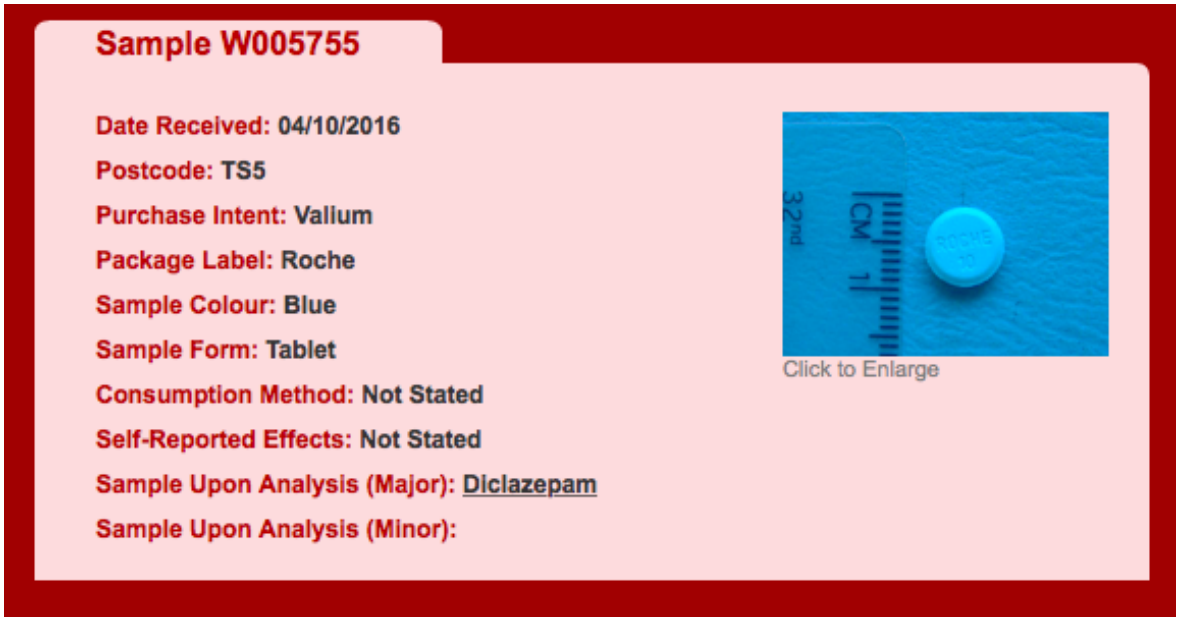

Figure 70: Tablet submission from the WEDINOS website in 2016.
(reproduced with permission from WEDINOS (https://www.wedinos.org)

7.3.1.3. Cause of death

The causes of death for the diclazepam positive cases were investigated for this study. Figure 71 displays the diclazepam post-mortem femoral blood concentrations and if it was included in the cause of death or not. Cases with a concentration of <0.005 mg/L were excluded from this graph.

There were 75 cases (21%) in this dataset where diclazepam was mentioned in any part of the cause of death, for example:

- *1a - Multiple organ failure; 1b - Hypoxic ischaemic brain damage; 1c - Cardiac arrest following fall; 2a - **Diclazepam** and alcohol intoxication*

was included as the word “diclazepam” featured despite it being in 2a and far down the list of contributing factors.

- *1a - Buprenorphine and benzodiazepine intoxication*

was not included as ‘diclazepam’ was not specifically named although benzodiazepine intoxication implies diclazepam may be implicated Other benzodiazepines are positive in this case.

There were 85 cases where diclazepam was not mentioned. The graph in Figure 71 shows there appears to be no relationship between concentration and the inclusion in the cause of death. There is almost even split between cases included and not included within the cause of death. It should be noted that there were several pathologists working on these cases in three separate areas of the country, this is likely to lead to difference of opinion and they will all interpret the significance of diclazepam differently. There are different categories of deaths included within the graph and are not all considered drug deaths, some would be hangings for example. The lack of published literature in this subject adds to this issue. The six cases clustered together at the top of the chart within the red circle are detailed in Table 64. These six cases stand out as they are above the main cluster of cases and three have diclazepam implicated in the cause of death and three have not. Five out of the six cases are from the SFIU West region and one from the SFIU East region - five different pathologists worked on these cases so the final cause of death decision is not being skewed by one particular pathologists opinion for these six cases. The details of case 3 are discussed in section 7.3.1.4 of this chapter.

Table 64: Case details for the 6 cases with the highest diclazepam concentration over the 6-year period (2013-2018) investigated

	Concentration (mg/L)						
Case	**Diclazepam**	**Delorazepam**	**Lormetazepam**	**Cause of Death**	**Sex**	**Age**	**Other drug findings**
1	0.15	0.69	0.07	1a - Heroin, gabapentin, diclazepam and methadone intoxication	F	32	Methadone, gabapentin, morphine, codeine, 6-MAM in urine
2	0.17	1.5	0.09	1a - Diclazepam, codeine and gabapentin intoxication	F	38	Paracetamol, gabapentin, pregabalin, codeine, morphine
3	0.211	0.23	0.041	1a -Methadone, alcohol and diclazepam intoxication	M	43	Alcohol,methadone, mirtazapine,THC and metabolite, diazepam and metabolite See 7.3.1.4
4	0.147	0.38	0.024	1a - Heroin, methadone and benzodiazepine intoxication	M	29	Alcohol, DMD, morphine, codeine, 6-MAM in blood and urine
5	0.14	Present	Present	1a Multi-drug toxicity	M	40	Cocaine metabolite-benzolyecogonine, mirtazapine, morphine, gabapentin and etizolam
6	0.089	0.11	0.02	1a Multi-drug toxicity	M	26	Low alcohol in blood, diazepam and metabolites, citalopram, paracetamol, THC and metabolite, morphine, codeine and 6-MAM in blood and urine

All six cases are drug related and cases 4 to 6 may not specifically name diclazepam but they do acknowledge combined drug use as the mechanism leading to the death.

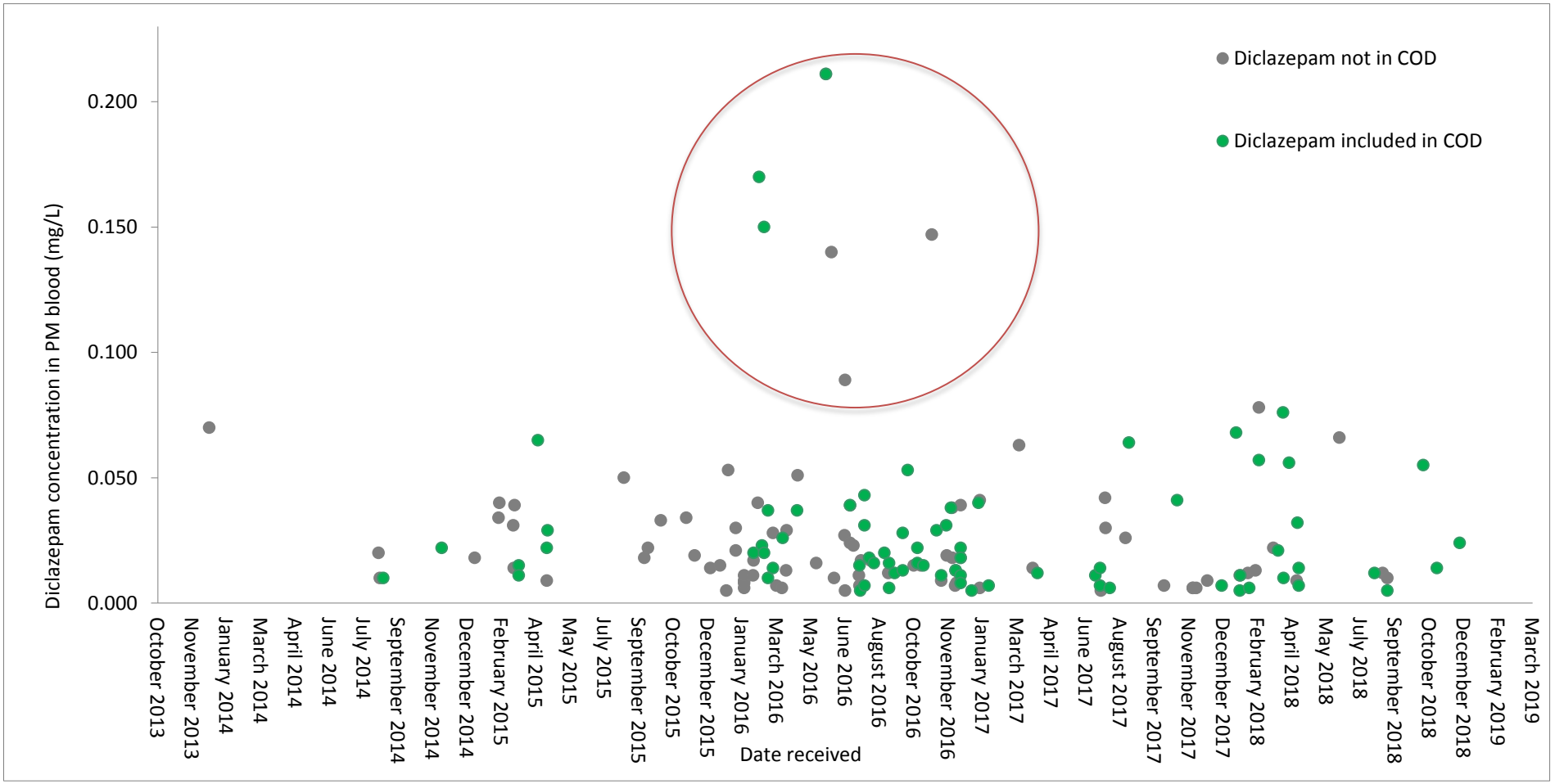

Figure 71: Diclazepam concentrations and role in cause of death

Each cause of death in this dataset was categorised into ten categories. The categories are shown in Table 65. The author was responsible for categorising these based on the words used in the cause of death. If a drug or intoxication was mentioned in any part of the cause of death then it was included in the drug related/intoxication category. If drugs *and* alcohol was mentioned this was counted as drug-related/intoxication and not “alcohol related.” The alcohol related category was for when alcohol only was mentioned with no other substances. The death fell into the category of “natural” when no intoxication was mentioned and no additional trauma was a factor. Examples of natural deaths included in this study’s categories were epilepsy, cirrhosis of the liver with no mention of alcohol or other substances and cardiac causes of death. Natural death is often a complex category for pathologists and there can be inconsistencies in how different pathologists would report a death with a combination of trauma and natural disease. (Roberts, Gorodkin and Benbow, 2000) Categories “drowning” and “immersion in water” are separate as immersion in water suggests there is no proof of drowning and death could have occurred due to another mechanism such as hypothermia. A system for categorising deaths where drugs are detected in the post-mortem blood was devised by Druid and Holmgren, this split the deaths into three groups designated as A, B and C. (Druid and Holmgren, 1997) Group A is a single drug intoxication death; this is not an expected scenario with benzodiazepines due to their relative safe nature when used alone. Group B is death by intoxication with one or more substances and/or alcohol and group C deaths are unrelated to the drug(s) detected within the post-mortem blood e.g. hanging. The categories in Table 65 are broken further down but mostly fall into group C.

Table 65: Cause of death categories used in this study

Drug related/intoxication
Natural
Stab wounds
Hanging /asphyxia
Unascertained
RTA (including pedestrian)
Alcohol related
Drowning
Immersion in water
Fire

The vast majority of diclazepam positive cases fell into the drug-related/intoxication category (83%) when the cause of death was considered in this way or group B using the Druid and Holmgren system. Figure 72 displays the cause of death when spilt into the categories in Table 65. Heroin was named in the cause of death in 25% of diclazepam positive cases. There are six cases, which have been excluded from the cause of death data for the diclazepam cases, as the author was unable to confirm the official cause of death.

The mechanism of death can be complex and multifaceted especially in drug users, this data does show that diclazepam is present in drug deaths but its significance is not entirely clear. The poly-drug use makes this difficult as well as the health issues drug users are at high risk of exposure to.

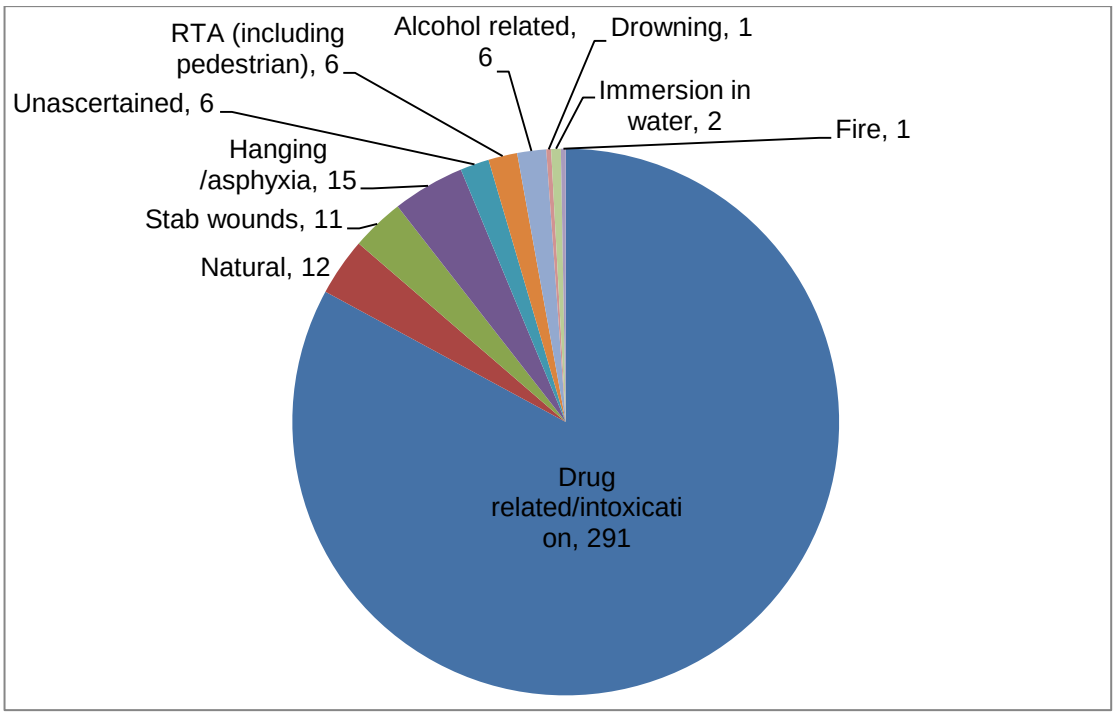

Figure 72: Cause of death categories for diclazepam positive cases over a 6-year period (2013-2018)

7.3.1.4. Case example

The highest diclazepam post-mortem femoral blood concentration detected in this study was in the case of a 43-year-old man who had a hepatitis C infection as well as a history of substance abuse. He told his doctor that he used cannabis and diazepam every night before bed. At the time of his death he was prescribed methadone, mirtazapine, levothyroxine (used in the treatment of hypothyroidism), omeprazole (used to decrease stomach acid) and amitriptyline. He was found face down in his bed with decomposition changes particularly to his skin and eyes. A search of the area found cannabis, dihydrocodeine tablets along with “street valium” consisting of over 100 blue tablets containing the marking “NTZ” and some yellow tablets with a “MSJ” marking. NTZ markings on blue tablets have been confirmed to contain etizolam in Scotland. (Scotland, 2016) However this may not represent what every blue “NTZ” tablet contains. The toxicological analysis revealed the following in the blood:

- Alcohol – 111 mg/100 mL
- Methadone – 0.71 mg/L
- Mirtazapine – 0.29 mg/L
- Diclazepam – 0.211 mg/L
- Delorazepam – 0.225 mg/L
- Lormetazepam – 0.041 mg/L
- Lorazepam – 0.01 mg/L
- THC and metabolite present
- Diazepam and desmethyldiazepam - <0.10 mg/L for both

Mirtazapine is known to cause drowsiness, alcohol and use of benzodiazepines is not recommended when mirtazapine is prescribed due to the excessive sedation that may occur. (Hartmann, 1999) The pathologist concluded that the moderate alcohol concentration in combination with methadone and diclazepam led to dangerous sedation and respiratory depression causing this man's death. They noted that the low concentration of diazepam and metabolite may also have contributed to the sedative effect, but were unlikely to be a major contributory factor in causing death. The cause of death was given as: *1a: Methadone, alcohol and diclazepam intoxication.*

7.3.2. Flubromazepam results

Flubromazepam was detected in 18 post-mortem cases over the six-year period included in this study. One positive was a case where only liver had been received and is therefore excluded from some results given. There were no flubromazepam positives detected in 2013 or 2018. Most flubromazepam positives were detected in 2017 (n=8) followed by 2016 (n=7).

7.3.2.1. Demographics

There were 15 males and 3 females that gave positive flubromazepam positive results in post-mortem cases in the six years examined. The mean age of this group was 37-years old (range, 28-51 years old). The mean age for males was 39-years old

and 32-years old for females. The number of cases positive for flubromazepam in each age range is presented in Figure 73.

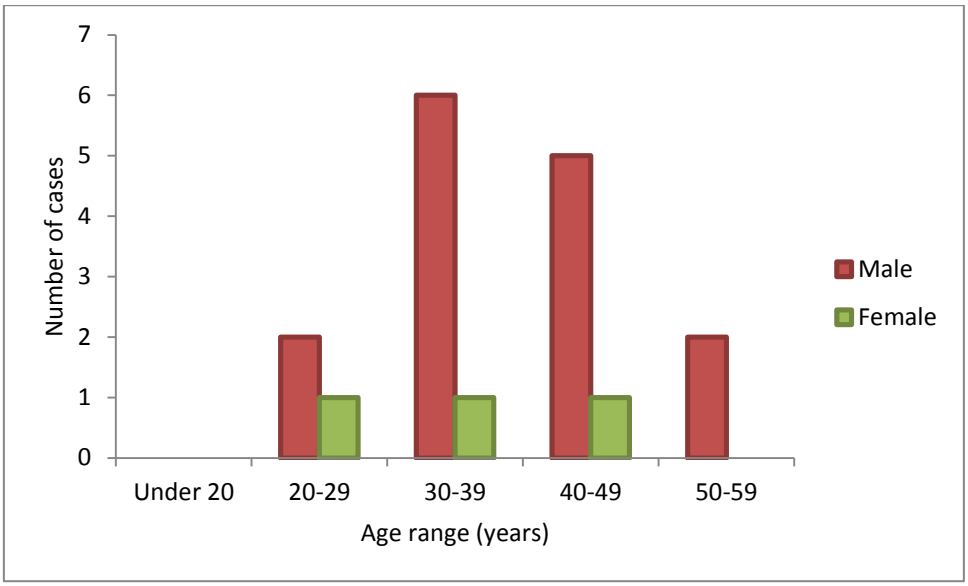

Figure 73: The range of ages for males and females in flubromazepam positive cases over a 6-year period (2013-2018)

7.3.2.2. Toxicological findings

The mean and median concentration in the post-mortem femoral blood was 0.74 mg/L and 0.66 mg/L for flubromazepam, respectively. Figure 74 shows the range of flubromazepam post-mortem blood concentrations detected; the graph shows that in most cases flubromazepam was above 0.20 mg/L. A more appropriate calibration range should be considered for the quantification of flubromazepam. A self-administration study found that the peak serum concentration was 0.078 mg/L six hours after the ingestion of a 4 mg capsule by a 43-year-old male. (Moosmann *et al.*, 2013a)

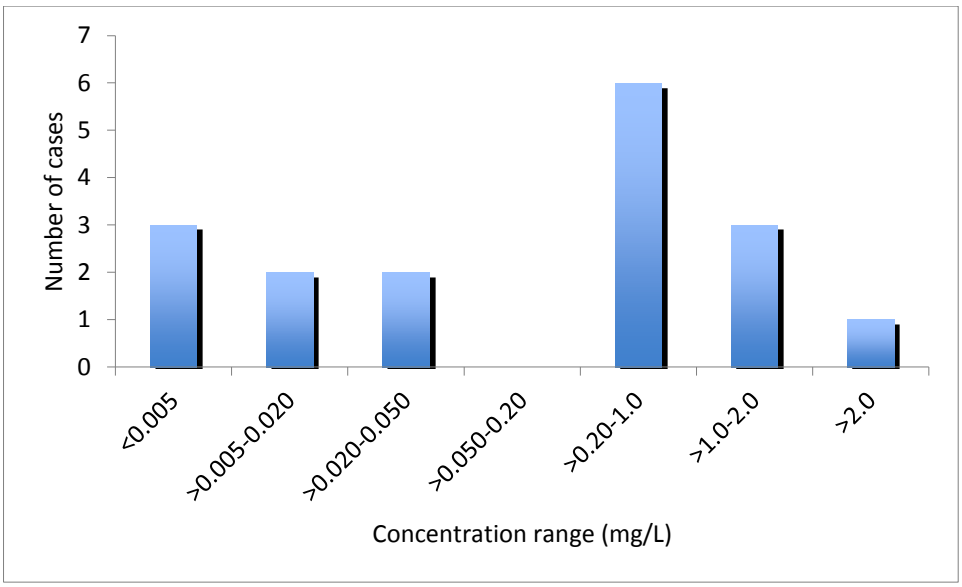

Figure 74: The range of flubromazepam concentrations detected

Pyrazolam was also detected in four of the flubromazepam positive post-mortem cases; diclazepam was detected in four cases and delorazepam was detected in seven cases. This demonstrates that the users of flubromazepam often took other designer benzodiazepines. The study by Høiseth *et al* collated concentrations in living offenders and they found 24 positive flubromazepam cases. These cases had a median blood concentration of 0.055 mg/L; the lowest concentration detected was 0.0047 mg/L and the maximum was 1.2 mg/L. (Høiseth, Tuv and Karinen, 2016) The FTS post-mortem data gathered in this study shows four cases with concentrations higher than 1.0 mg/L, see Figure 74. Høiseth *et al* noted that a 37-year-old male who had a flubromazepam blood concentration of 0.60 mg/L with no other drugs detected was “mildly impaired.” (Høiseth, Tuv and Karinen, 2016) An article published in 2018 by Koch *et al* described a lethal case of U-47700 and flubromazepam intoxication. (Koch *et al.*, 2018) U-47700 is a synthetic opioid and is considered an NPS. (Koch *et al.*, 2018) A 24-year-old male was hospitalised after consuming both U-47700 and flubromazepam, his flubromazepam serum concentration was 0.83 mg/L 42 minutes after hospital admission, this fell to 0.28 mg/L in serum three days later. Six days after hospital admission the patient died after life support was removed. (Koch *et al.*, 2018) Six cases in the FTS post-mortem flubromazepam positive dataset in this study were above 0.80 mg/L, see Figure 74. U-47700 alone could have possibly caused death

however the combination of these two drugs is very dangerous. As drug distribution can vary widely between blood and serum, concentrations in these sample types are not always comparable. (Launiainen and Ojanperä, 2014)

There were three flubromazepam positive cases, that were also positive for flubromazolam, see section 1.5.1 in chapter 1 for the structural difference of these two compounds. The first case was from 2015 and was 33 year old male who had the following post-mortem blood concentrations:

- Flubromazolam - 0.27 mg/L
- Flubromazepam - 1.6 mg/L
- Lorazepam - <0.005 mg/L
- Delorazepam – 0.021 mg/L
- Alcohol – 25 mg/100 mL

The cause of death was given as *1a: Mixed benzodiazepine (flubromazepam, flubromazolam, delorazepam, lorazepam) and alcohol toxicity.*

A case from Poland published by Łukasik-Głębocka *et al* (Łukasik-Głębocka *et al.*, 2016) described a 27-year-old male who had a variety of serious symptoms including deep coma and acute respiratory failure. Analysis of his serum sample detected flubromazolam at a concentration of 0.059 mg/L, this serum sample was thought to have been drawn around 19 hours after a 3 mg dose. No other drugs were detected. The patient survived due to the medical intervention he received. (Łukasik-Głębocka *et al.*, 2016)

The other two FTS flubromazepam and flubromazolam-positive cases both had a concentration of <0.005 mg/L for both flubromazolam and flubromazepam in post-mortem blood. One case was a 24-year-old female; the cause of death was concluded as *1a Amitriptyline and venlafaxine intoxication*. The other case was a 44-year-old male; the cause of death was concluded as *1a Ischaemic heart disease; 1b Cardiac enlargement and coronary artery atheroma.*

The concentrations documented in the articles by Koch *et al* (Koch *et al.*, 2018) and Łukasik-Głębocka *et al* (Łukasik-Głębocka *et al.*, 2016) suggest that the flubromazepam and flubromazolam concentrations found in the 2015 case described

in this study are particularly high, however this is a comparison of blood and serum concentrations, which as stated before cannot be reliably compared due to the widely varying distribution of drugs between sample types. Comparing ante-mortem and post-mortem concentrations also cannot be reliably compared due to factors such as post-mortem redistribution.

7.3.2.3. Cause of death

The cause of death attributed to the flubromazepam positive cases were investigated for this study. There were 7 cases in which flubromazepam was mentioned in the cause of death. The flubromazepam post-mortem blood concentrations and cause of deaths are shown in Table 66. These fall into group B using the Druid and Holmgren system.

Table 66: Flubromazepam post-mortem femoral blood concentrations in cases where flubromazepam is mentioned in cause of death

Flubromazepam (mg/L)	Cause of death
1.60	1a-Mixed Benzodiazepine (Flubromazepam, Flubromazolam, Delorazepam, Lorazepam) and Alcohol Toxicity
1.30	1a-Dihydrocodeine, Flubromazepam, Pyrazolam and Hydrocodone toxicity
2.30	1a-Methdone, flubromazepam and gabapentin intoxication
0.85	1a-Heroin, morphine, etizolam and flubromazepam intoxication
1.10	1a-Morphine and flubromazepam intoxication
0.42	1a-Flubromazepam, alcohol intoxication and cocaine
0.01	1a-Heroin, gabapentin, methadone, etizolam and flubromazepam intoxication; 2- ischaemic heart disease with cardiac enlargement

The cases where flubromazepam was included in the cause of death has a mean concentration of 1.08 mg/L and a median of 1.10 mg/L which is higher than the overall mean and median which was 0.74 mg/L and 0.66 mg/L respectively.

Table 67: Flubromazepam post-mortem femoral blood concentrations in cases where flubromazepam is not mentioned in cause of death

Flubromazepam (mg/L)	Cause of death
0.80	1a-Heroin and benzodiazepine toxicity
0.69	1a-Tramadol and venlafaxine intoxication
0.663	1a-Unascertained
<0.005	1a-Amitriptyline and venlafaxine intoxication
0.46	1a-Hanging
<0.005	1a-Ischaemic heart disease; 1b Cardiac enlargement and coronary artery atheroma
0.013	1a- Ischaemic heart disease and possible drug toxicity 2-Fatty degeneration of the liver
0.029	1a-Dihydrocodeine, tramadol, morphine, gabapentin and benzodiazepine intoxication
0.59	1a-Methadone, morphine and benzodiazepine intoxication
<0.005	1a Drowning 2a Alcohol and multi-drug toxicity 2b Epilepsy

Table 67 displays the flubromazepam-positive cases where flubromazepam was not included in the cause of death. The cases where flubromazepam was not included in the cause of death have a mean concentration of 0.59 mg/L and a median of 0.59 mg/L which is lower than the overall mean and median of 0.74 mg/L and 0.66 mg/L respectively however this is a small dataset and there is no significant difference in the concentrations included in the cause of death compared to the concentrations not included (*p*-value=0.06).

The same categories of death were applied to the post-mortem flubromazepam cases as detailed in 7.3.1.3. Figure 75 shows the categories of death of the flubromazepam positive cases. The vast majority of flubromazepam positive cases fell into the drug-related/intoxication category (76%) (Group B using the Druid and Holmgren system) when the cause of death was considered in this way. There were only three flubromazepam positive cases that included heroin in the cause of death.

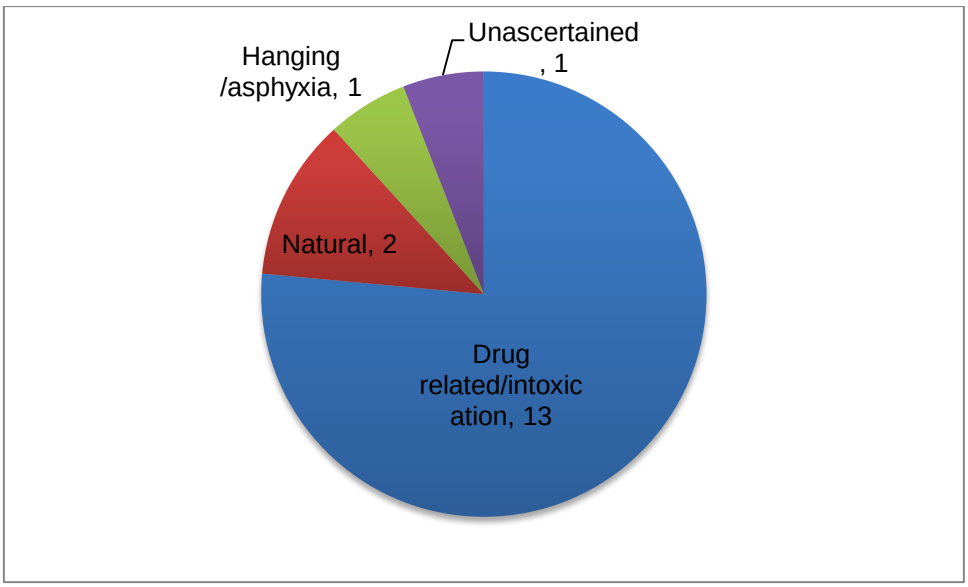

Figure 75: Cause of death categories for flubromazepam positive cases over a 6-year period (2013-2018)

7.3.2.4. Case example

The highest flubromazepam concentration detected in this study was in the case of a 51-year-old man who had a history of schizophrenia, depression and drug abuse. He was known to use heroin and “street valium.” He was prescribed methadone, sertraline and the antipsychotic drug pericyazine, at the time of his death. He was found slumped and unresponsive on his bed with post-mortem staining to his face. The previous night he was witnessed consuming around 30 “street valium” tablets and 20 gabapentin tablets. The police search of the area found a small empty polythene bag, which they noted might have contained drugs. It is not known if any forensic testing was carried out on any residue from the polythene bag. The toxicological analysis detected the following in the post-mortem femoral blood:

- Methadone - 1.3 mg/L
- Sertraline - 0.27 mg/L
- Gabapentin - 18 mg/L
- Flubromazepam - 2.3 mg/L
- Pyrazolam - <0.005 mg/L
- Diazepam - <0.05 mg/L, Desmethyldiazepam – 0.07 mg/L

The pathologist determined this case to be a drug-related death with the low concentrations of diazepam and metabolite and pyrazolam as well as the therapeutic concentration of the sertraline possibly having a cumulative effect with the high concentration of methadone, flubromazepam and moderate concentration of gabapentin. The cause of death was concluded as *1a: Methadone, flubromazepam and gabapentin intoxication.*

7.3.3. Pyrazolam results

Pyrazolam was detected in 9 post-mortem femoral blood cases over the six-year period examined. Like flubromazepam there were no pyrazolam positives detected in 2013 or 2018. Most pyrazolam positives were detected in 2016 (n=4) followed by 2017 (n=3). According to anecdotal reports pyrazolam has low sedation effects and has a low recreational value in general. (Manchester *et al.*, 2018) This may explain why there are a low number of positives in the post-mortem femoral blood, as it may be a less attractive drug to the Scottish drug users than other benzodiazepines.

7.3.3.1. Demographics

There were 7 males and 2 females, who were positive for pyrazolam in the six years examined. The mean age of this group was 28 years old (range, 20 to 51-years old).

7.3.3.2. Toxicological findings

The mean and median concentrations in the post-mortem blood were 0.209 mg/L and 0.033 mg/L for pyrazolam, respectively. Figure 76 shows the range of pyrazolam concentrations detected; the graph shows how the pyrazolam concentration was below 0.050 mg/L in most cases. There is one case with a concentration of 1.1 mg/L, which has skewed the mean concentration, and therefore the median concentration is a more appropriate reflection of the concentrations found in this dataset. A self-administration study found a peak serum concentration was 0.051 mg/L three hours after the ingestion of a two 0.5 mg tablets. (Moosmann *et al.*, 2013b) The study by Høiseth *et al.* collated concentrations in living offenders and they found 1 positive pyrazolam case, which had a blood concentration of 0.074 mg/L. (Høiseth, Tuv and Karinen, 2016) An article published in 2018 noted that pyrazolam had been detected

in urine samples in 33 instances from 2012 to 2016 in individuals presenting as intoxicated to Swedish emergency departments, no concentration data was included in the article. (Bäckberg *et al.*, 2019)

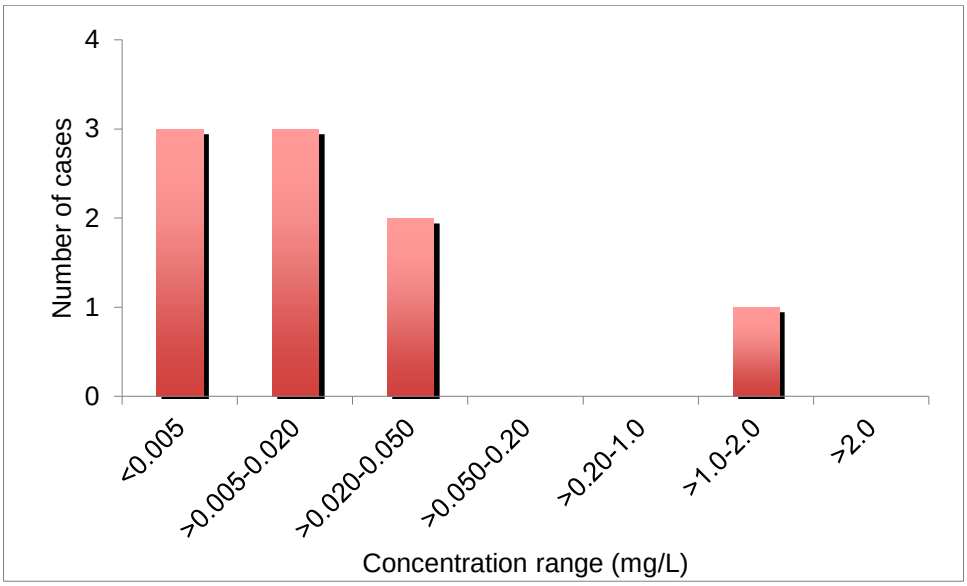

Figure 76: The range of pyrazolam concentrations detected in post-mortem femoral blood samples over a 6-year period (2013-2018)

Flubromazepam was also detected in six of the pyrazolam positive post-mortem cases; diclazepam was detected in one case.

7.3.3.3. Cause of death

The causes of death attributed to the pyrazolam-positive cases were investigated for this study. There were 3 cases in which pyrazolam was mentioned in the cause of death. The pyrazolam post-mortem blood concentrations and causes of death are shown in Table 68.

Table 68: Pyrazolam post-mortem femoral blood concentrations in cases where pyrazolam is mentioned in cause of death

Pyrazolam (mg/L)	Cause of death
0.070	1a-Ethylphenidate, methoxphenidine, morphine, pyrazolam and etizolam intoxication
1.100	1a-Dihydrocodeine, Flubromazepam, Pyrazolam and Hydrocodone toxicity
0.012	1a-Heroin, gabapentin, etizolam, pyrazolam, flubromazolam intoxication

The cases where pyrazolam is included in the cause of death has a mean concentration of 0.39 mg/L and a median of 0.070 mg/L which is higher than the overall mean and median which were 0.209 mg/L and 0.033 mg/L, respectively.

Table 69: Pyrazolam post-mortem femoral blood concentrations in cases where pyrazolam is not mentioned in cause of death

Pyrazolam (mg/L)	Cause of death
0.028	1a-Heroin and methadone intoxication
0.037	1a-Morphine and alcohol intoxication
<0.005	1a-Ischaemic heart disease 1b-Cardiac enlargement and coronary artery atheroma
0.008	1a-Multi-drug toxicity 2-Anomalous origin of the coronary arteries
<0.005	1a-Methdone, flubromazepam and gabapentin intoxication
<0.005	1a-Heroin, morphine, etizolam and flubromazepam intoxication

Table 69 displays the pyrazolam-positive cases where pyrazolam was not included in the cause of death. The cases where pyrazolam was not included in the cause of death have a mean concentration of 0.028 mg/L and a median of 0.028mg/L, which is lower than the overall mean, and median that were 0.209 mg/L and 0.033 mg/L, respectively. This is a very small dataset that is skewed by the 1.1 mg/L result. There was no significant difference found in the concentrations included in the cause of death compared to the concentrations not included (*p*-value =0.39).

The same categories of death were applied to the post-mortem pyrazolam cases as detailed in section 7.3.1.3 Every case except one of the pyrazolam-positive cases fell into the drug-related/intoxication category (89%) (Group B using the Druid and Holmgren system) when the cause of death was considered in this way. There were three pyrazolam positive cases that included heroin in the cause of death.

7.3.3.4. Case example

This case was a 30-year-old male who was found slumped on his bed in a state of advanced decomposition. He had a syringe in his leg and a bag of citric acid in his hand. He was not prescribed any medication at the time of his death but had previously admitted addiction to heroin, “street diazepam”, alcohol and ecstasy to his doctor. A search of the area found uncapped needles, burnt spoons, brown powder, burnt foil and empty “legal high” packets. The packets appeared to be pyrazolam, as well as deschloroetizolam and clonazolam. The toxicological analysis revealed the following in the post-mortem femoral blood:

- Alcohol – 155 mg/100 mL
- Morphine – 0.21 mg/L
- Pyrazolam – 0.037 mg/L

Chest blood was also submitted and was positive for morphine, codeine and pyrazolam. Deschloroetizolam and clonazolam were included in the analysis, and were found to be negative in both chest and femoral blood.

The pathologist in this case did not include pyrazolam in the cause of death, which was concluded as *1a: Morphine and alcohol intoxication*.

7.3.4. Other benzodiazepines in post-mortem samples

7.3.4.1. Etizolam

The most prevalent designer benzodiazepine in the post-mortem cases submitted to FTS is etizolam; from October 2013 to December 2017 it was detected in 993 cases (7.3%). Etizolam in post-mortem cases will not be explored any further in this study.

7.3.4.2. Alprazolam

There have been 232 alprazolam-positive cases from 2016 to 2018 in the post-mortem cases submitted to FTS. The mean age in these cases was 39 years with 77% of cases being male (n=178) and 23% female (n=54). During this time period, not every case submitted to FTS was analysed for alprazolam. The inclusion of alprazolam is dependent on the case circumstances, for example if it is mentioned in the police death report. Alprazolam may also be picked up within another analysis, such as an interfering peak with a different benzodiazepine method or a library spectrum match within the basic drug GC-MS analysis. Therefore true prevalence cannot be fully determined from this data.

Figure 78 shows the number of cases where alprazolam was detected in post-mortem blood from 2013 to 2018.

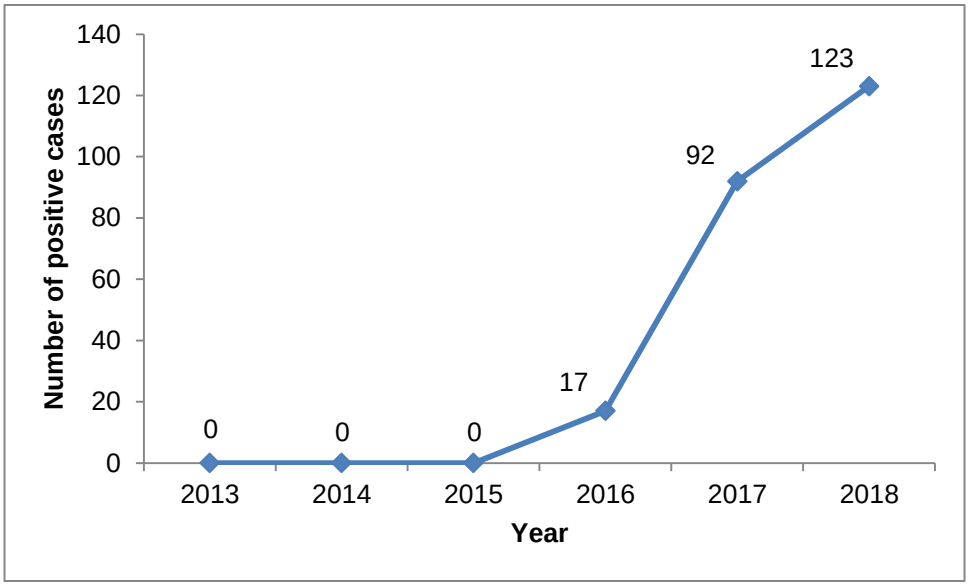

Figure 77:Alprazolam positive cases in FTS post-mortem casework

Figure 77 shows an increase in alprazolam in post-mortem cases over the last three years, which is in contrast to diclazepam, which has decreased since 2016.

The mean concentration of alprazolam in this dataset was 0.179 mg/L. A Swedish study published in 2013 compared the alprazolam concentrations found in post-mortem samples with those found in impaired drivers. (Jones and Holmgren, 2013) They discovered the mean concentration detected in deaths attributed to drug intoxication was 0.10 mg/L with the range 0.02 - 1.6 mg/L. The mean concentration in other causes of death was 0.08 mg/L with the range 0.02 - 0.9 mg/L. Interestingly the concentrations in blood from impaired drivers were very similar to those found in the post-mortem samples. The mean concentration in impaired drivers was 0.08 mg/L with the range 0.02 - 3.9 mg/L. (Jones, Mogali and Comer, 2012) This may be explained by users developing tolerance which commonly occurs with benzodiazepine use, this in turn results in increased dosage. (Ashton, 2005) Tolerance is thought to occur when the GABA receptor becomes desensitised to the repeated use of benzodiazepines leading to dependence. Severe withdrawal symptoms occur when a dependent user reduces their dose or stops taking benzodiazepines.

7.3.4.3. Designer benzodiazepines in 2019 post-mortem samples

In the first eight months of 2019 there were 25 delorazepam positive cases, 14 of which have also tested positive for diclazepam and 8 for lormetazepam. It appears that diclazepam is steadily becoming less prevalent in post-mortem samples in Scotland; this could be due to lack of availability as a consequence of the Psychoactive Substances Act 2016 and the availability or preference for other drugs such as etizolam. The typical concentrations found in the 2019 positive samples have remained consistent with those of the previous six years. Table 70 displays the mean and median concentrations for the 2019 cases.

Table 70: Diclazepam and metabolites mean and median post-mortem femoral blood concentrations in 2019 post-mortem cases

	Diclazepam	**Delorazepam**	**Lormetazepam**
Mean concentration (mg/L)	0.026	0.106	0.015
Median concentration (mg/L)	0.017	0.028	0.006

One positive case of flubromazolam was detected in 2019 however the deceased was found heavily decomposed at the start of the year so the drug could have been ingested at the end of 2018. This case was a 34-year-old male who had a history of drug abuse. As the blood sample provided was central blood the concentrations of drugs found were not interpreted. Central blood tends to give inaccurate, usually elevated concentrations of drugs due to post-mortem redistribution. (Pounder and Jones, 1990) Morphine and codeine were detected in the central blood in addition to cyproheptadine, which is an antihistamine. The EMCCDA received a report from Ireland suggesting that cyproheptadine may be in fake Xanax tablets (alprazolam). (*European Monitoring Centre for Drug and Drug Addiction, European Database on New Drugs.*) The urine sample in this case was positive for 6-monoacetylmorphine and alcohol.

7.4. Conclusion

This study gives some idea of the typical concentrations seen in the post-mortem blood of users of flubromazepam, pyrazolam and diclazepam in particular. This study has shown that designer benzodiazepines are present in drug related deaths and are

found in combination with other drugs. Due to poly-drug use it is difficult to assess the significance of designer benzodiazepines in an individuals' death, but it is very likely it has a cumulative effect when combined with other CNS depressants. Over the six-year period examined, diclazepam suddenly became a very popular drug in the drug-using population in Scotland (especially in 2016), but is now steadily in decline and is being replaced with the likes of alprazolam. Flubromazepam, pyrazolam and flubromazolam had a small part to play in the designer benzodiazepine using population but never reached the popularity of etizolam and diclazepam.

8. Conclusions

The abuse of benzodiazepines has been a problem in Scotland for decades. The introduction of designer benzodiazepines from 2010 onwards exacerbated this; it led to issues such as how these newly abused drugs contribute to a drug related cause of death, how treatment services deal with the users and users being unaware of what benzodiazepines they are taking. This is especially dangerous as benzodiazepine users often take “supratherapeutic” doses. This work investigated the use of designer benzodiazepines as well as traditional prescription benzodiazepines in sub-populations in Scotland.

This work has shown the Immunalysis® Benzodiazepine ELISA kit can positively identify phenazepam, etizolam, diclazepam, delorazepam, pyrazolam and flubromazepam in blood. This is vital to know for a routine forensic toxicology laboratory as it can reduce the number of samples required for confirmation and quantitation. Positive samples are therefore not likely to be missed at the screening step but there is a lack specificity to help analyse what particular benzodiazepine(s) is present. PM samples are often decomposed which may have a deleterious effect on drug detection.

A quantitative LC-MS/MS, SPE method for the detection of diclazepam and two of its metabolites (delorazepam and lormetazepam) plus flubromazepam and pyrazolam was validated. The method was fit for purpose. The concentration range (0.005-0.20 mg/L) selected for these drugs in blood gave acceptable linearity. The method is specific and selective and demonstrated no carryover at the concentrations tested. Bias and precision were acceptable. Matrix effects were found to be acceptable for all analytes and within the ±25% criterion for %ME and <15% for precision. Recovery and process efficiency was suitable for diclazepam and delorazepam, it was suboptimal for lormetazepam, pyrazolam and flubromazepam however the LOD of 2 ng/mL was sufficient for these analytes. All analytes were stable in the autosampler up to 48 hours at 16-24 °C.

A qualitative LC-MS/MS, LLE method to detect 22 benzodiazepines compounds in urine was developed and validated. This method was fit for purpose, it had a limit of detection range of 2 ng/mL - 6 ng/mL, an acceptable range of matrix effects and

recovery was acceptable with the exception of pyrazolam recovery. The method is specific and demonstrated no carryover at the concentration tested. All analytes were stable in the autosampler up to 36 hours at 16-24 °C.

The quantitative LC-MS/MS, SPE method was used to analyse 1691 post-mortem blood samples over a six-year period. There was a total of 369 cases positive for designer benzodiazepines in blood (22% overall), 354 of these were diclazepam positive (21% overall).

The 369 total positive cases also included 18 flubromazepam positive cases (1% of all cases analysed), 7 of which were also diclazepam positive cases and 4 were positive for pyrazolam.

The 369 total positive cases also included 9 pyrazolam positive cases (0.5% of all cases analysed), 1 of which was also diclazepam positive.

This study also provided information on the typical concentrations seen in the post-mortem femoral blood of users of flubromazepam, pyrazolam and diclazepam.

Diclazepam was detected in 212 cases and gave a median concentration of 0.017 mg/L (n=157, 0.005 – 0.211 mg/L). Delorazepam was detected in 339 cases and gave a median concentration of 0.043 mg/L (n=311, 0.005 – 1.50 mg/L). Lormetazepam was detected in 144 cases and gave a median concentration of 0.010 mg/L (n=85, 0.005 – 0.18 mg/L).

The findings in this study answered a gap in the knowledge regarding post-mortem blood concentrations of diclazepam and two of its metabolites. It showed that delorazepam was detected at higher blood concentrations than diclazepam and lormetazepam, and gave the longest window of detection in post-mortem femoral blood. This study has shown that these analytes are present in drug related deaths and are found in combination with other drugs. Due to poly-drug use it is difficult to assess the significance of designer benzodiazepines in an individuals' death, but it is very likely it has a cumulative effect when combined with other CNS depressants. This data can be utilised as a reference for toxicologists and pathologists when interpreting the presence of these drugs and their metabolites in post-mortem cases. It is most probably due to the lack of knowledge of these drugs that this study found

there was no relationship between the drug concentration and the inclusion in the cause of death. Different pathologists assigning the cause of death over the caseload also adds to inconsistencies. This dataset found there were more males positive for diclazepam in post-mortem cases than females and the mean age found in this study remains consistent for the cases from 2014 to 2018, (range, 18-75 years old). The mean age for males was 40-years old and 41-years old for females. This is in-line with the drug-related death statistics, most DRDs in Scotland are individuals between 25-44 years old and 75% are male. The diclazepam dataset displayed an increasing trend in popularity, which reached its peak in 2016; the PSA ended the high street and online availability and while diclazepam did not disappear its' presence in PM cases declined from 2016.

Flubromazepam and pyrazolam were also detected in PM cases and the blood concentrations will also provide a good resource in aiding toxicological interpretation, however they were detected on a much smaller scale than diclazepam. Flubromazepam was detected in 18 cases and gave a median concentration of 0.66 mg/L (n=15, 0.01 – 2.30 mg/L). Pyrazolam was detected in 9 cases and gave a median concentration of 0.033 mg/L (n=6, 0.008 – 1.10 mg/L). The flubromazepam blood concentrations were much higher than diclazepam and perhaps a more appropriate calibration range should be applied to their analysis. The demographics for these two drugs are also in agreement with the drug-related death statistics. The concentrations of flubromazepam are higher than most of the concentrations reported in the literature, however there is not a great deal of studies in which to compare. It may be the case that the high doses ('supratherapeutic") that Scottish benzodiazepine users are reported to take accounts for this. Pyrazolam was only detected in 9 post-mortem cases over the six-year period examined suggesting it is not a popular drug of abuse in Scotland. One explanation for this is that pyrazolam is anecdotally reported to have low recreational value and low sedation making it an unlikely choice amongst Scottish drug users.

The secondary aim of this research was to determine if individuals from high-risk populations are using benzodiazepines. These cohorts provided unpreserved urine samples for analysis and were tested for 22 analytes to provide a more comprehensive result. The qualitative LC-MS/MS, LLE urine method was used to

analyse 73 individuals under a DTTO from the Scottish Drug Court, 95 patients receiving forensic psychiatry treatment from the NHS GGC FD and 725 samples from individuals being admitted or liberated from SPS facilities. The SDC and FD cohorts were found to be largely compliant with the conditions imposed upon them, concerns that their testing method was not detecting designer benzodiazepines could not be fully answered as no designer benzodiazepines other than 3 instances of diclazepam metabolites and 1 instance of etizolam and metizolam in the SDC cohort were found. These four samples were also positive for diazepam metabolites so would have tested positive in the SDC screen. This study found that diazepam was being commonly used in the SDC. The forensic psychiatry results showed a population who did not appear to be using benzodiazepines.

The third urine sample population evaluated was the SPS samples. The SPS study showed the majority (59%) of participants were negative for all benzodiazepines tested. Diazepam and/or its metabolites (n=281) were the most commonly detected followed by diclazepam and/or its metabolites (n=59) then etizolam (n=22), 3-hydroxyphenazepam (n=21), metizolam (n=7), phenazepam (n=6) then pyrazolam (n=1) and flubromazepam (n=1). HMP Barlinnie had the highest number of diclazepam metabolite positives - 21% of admission samples. Other designer benzodiazepines did not have a high presence overall, at 3% for etizolam, the second most common designer benzodiazepine after diclazepam. The vast majority (73%) of positive SPS samples for designer benzodiazepines showed use of one benzodiazepine drug. Admission SPS samples were more likely to be positive than liberation samples suggesting there is general abstinence of benzodiazepine use while in prison however the participation in this study was voluntary and this can skew the results. Despite this, this SPS study detected more positive samples than prison APT scheme that tested more samples overall. This emphasises why confirmation techniques are required and that a screening method should not be relied upon to provide an accurate assessment.

This research contains limitations and cannot be thought of as true prevalence studies as consent was required for the urine samples to be tested and the SDC and NHS GGC FD patients were aware of their test date. Therefore they could have

abstained from drug use prior to sampling. The SPS samples provide a snapshot but are not indicative of the general static inmate population.

Benzodiazepine urine analysis interpretation can be particularly complex, many metabolise to other benzodiazepines that are also parent drugs. In some instances the source of the analyte detected is not known, as there may be a number of possibilities. A particular challenge in this study was the source of 3-hydroxyphenazepam and metizolam especially since there is a lack of literature pertaining to both of their metabolism. There is sufficient evidence to argue that it is likely the 3-hydroxyphenazepam detected in the SPS samples is from phenazepam metabolism and not 3-hydroxyphenazepam consumption as the samples were collected before 3-hydroxyphenazepam had been reported as a designer benzodiazepine to the EMCDDA. Metizolam was reported in 2015 to the EMCDDA, the same year as the SPS study and after most of the SDC were collected. The source of the metizolam in some urine samples is unknown however metabolism from etizolam and in-source degradation from etizolam was ruled out. The absence of some metabolites, in particular alpha-hydroxyetizolam, was a limitation for the urine method used. However, the metabolism of most designer benzodiazepines is not well documented in the literature due to their novelty. For this same reason commercially available certified reference standards were not readily available at the time of the urine method development. Inclusion of diazepam and diclazepam metabolites within the method took priority over the less popular designer benzodiazepines.

Overall this research provides valuable information on the scale of benzodiazepine use in different populations, how popular a drug is and how this trend changes, as well as the typical designer benzodiazepine blood concentrations found in post-mortem cases which will ultimately aid the forensic toxicologists and forensic pathologists when interpreting concentrations found in their cases.

9. Further work

The ever-changing drug market means that routine analytical methods should be frequently reviewed and new compounds added for monitoring as required. This study has shown that users, inadvertently or not, have moved towards other benzodiazepines such as alprazolam when it was previously not being abused in Scotland. The dynamic nature of the drug market is a challenge for a laboratory to keep up to date and this can be practically difficult, utilising a screening method for a number of compounds makes this task slightly easier and negates the need to frequently quantitatively revalidate.

The methods detailed in this work could be improved upon; the addition of metabolites when they became available from reference standard suppliers would be beneficial and would give a wider time frame of detection. The addition of alpha-hydroxyetizolam to the urine method would be the most significant initial change and would likely identify some past use previously missed. The extraction methods could also be optimised or further developed in order to improve recovery. Pyrazolam in particular would benefit from a stability investigation and the use of acetonitrile in the solutions might improve the recovery. A thorough investigation into the presence of metizolam to determine its source within the positive samples should be carried out; the further monitoring of etizolam positives in urine samples and the ratios in which these are found may provide evidence of metizolam being produced from etizolam synthesis rather than metizolam consumption. The lack of information regarding metizolams metabolism hinders this as well as being unable to source the metabolites as a certified reference material.

Extending the cohorts to include work place testing or other patients under different forms of treatment would give additional valuable information on the scale of use. This could also reveal more trend information and chart any peaks and troughs in popularity. Testing every post-mortem case even as a screen would also give more complete data in terms of prevalence. This could then be confirmed quantitatively to add to the post-mortem blood typical concentration data. An investigation into the presence of lorazepam and its concentrations in blood for diclazepam positive cases should be carried out and would further add to the post-mortem data. In addition, repeating the projects with the same cohorts such as with the SPS or Scottish Drug

Court would be very interesting as it would give a direct comparison and could demonstrate if there has been a change in benzodiazepine drug trends.

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11. Appendices

11.1. Ethical approval to collect blank (drug free) urine

University of Glasgow

Dear Dr Hilary Hamnett

MVLS College Ethics Committee

Project Title: *Collection of blank biological samples*
Project No: *200160020*

The College Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study. We are happy therefore to approve the project, subject to the following conditions:

- Project end date:
- The data should be held securely for a period of ten years after the completion of the research project, or for longer if specified by the research funder or sponsor, in accordance with the University's Code of Good Practice in Research: (http://www.gla.ac.uk/media/media_227599_en.pdf)
- The research should be carried out only on the sites, and/or with the groups defined in the application.
- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.
- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely

Dr Terry Quinn

Terry Quinn
MD, FRCP, BSc (hons), MBChB (hons)
Senior Lecturer / Honorary Consultant

College of Medicine, Veterinary & Life Sciences
Institute of Cardiovascular and Medical Sciences
New Lister Building, Glasgow Royal Infirmary
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11.2. Ethical approval to test the Scottish Drug Court urine samples.

University of Glasgow | College of Medical, Veterinary & Life Sciences

8th May 2015

Dear Lauren O'Connor, Hazel Torrance, Denise McKeown, Jo McManus, Lynn Macdonald

MVLS College Ethics Committee

Project Title: Detecting drugs of abuse including NPS in urine samples
Project No: 200140101

The College Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study. It is happy therefore to approve the project, subject to the following conditions:

- Project end date: April 2016
- The data should be held securely for a period of ten years after the completion of the research project, or for longer if specified by the research funder or sponsor, in accordance with the University's Code of Good Practice in Research: (http://www.gla.ac.uk/media/media_227599_en.pdf)
- The research should be carried out only on the sites, and/or with the groups defined in the application.
- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.
- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely

Prof. Andrew C. Rankin
Deputy Chair, College Ethics Committee

Andrew C. Rankin
Professor of Medical Cardiology
BHF Glasgow Cardiovascular Research Centre
College of Medical, Veterinary & Life Sciences
University of Glasgow, G12 8TA
Tel: 0141 211 4833
Email: andrew.rankin@glasgow.ac.uk

11.3. Ethical approval to test the NHS GCC FD patient's urine samples.

WoSRES
West of Scotland Research Ethics Service

NHS
Greater Glasgow and Clyde

West of Scotland REC 3
Ground Floor – The Tennent Institute
Western Infirmary
38 Church Street
Glasgow G11 6NT
www.nhsggc.org.uk

Dr Richard Stevenson
Consultant
NHS Greater Glasgow and Clyde
Emergency Department
Glasgow Royal Infirmary
Glasgow
G4 0SF

Date 1st December 2015
Your Ref
Our Ref
Direct line 0141 211 2123
Fax 0141 211 1847
E-mail WOSREC3@ggc.scot.nhs.uk

Dear Dr Stevenson

Study title:	**Extended Urine Toxicology Screening of Forensic Psychiatric Patients**
REC reference:	**15/WS/0263**
IRAS project ID:	**187536**

Thank you for your email dated 30th November 2015. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 27 November 2015

Documents received

The documents received were as follows:

Document	*Version*	*Date*
Other [Email confirming additional conditions]		30 November 2015

Approved documents

The final list of approved documentation for the study is therefore as follows:

Document	*Version*	*Date*
Covering letter on headed paper [Covering letter]	1	15 October 2015
Other [Email confirming additional conditions]		30 November 2015
REC Application Form [REC_Form_26102015]		26 October 2015
Research protocol or project proposal [Protocol]	1.2	23 October 2015
Summary CV for Chief Investigator (CI) [RichardStevensonResarchCV]	1	

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices

at all participating sites.

15/WS/0263	Please quote this number on all correspondence

Yours sincerely

Liz Jamieson
REC Manager

Copy to: Dr Michael Barber, NHS Greater Glasgow and Clyde R&D

11.4. Scottish Drug Court Participant Information Sheet

University of Glasgow | Section of Forensic Medicine & Science

Participant Information Sheet

Detecting drugs of abuse including NPS in Urine Samples

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of the study?

This study is being performed as Drug Court clients often report using drugs but their urine-screening test is negative. This study will attempt to discover what substances are being used. It is suspected that many clients may be using New Psychoactive Substances (NPS). This may be because they are legal, to evade detection; or because of the increasing popularity and availability of these substances. Users may also believe that they have purchased a different drug to what they have actually received. The study will also gain valuable data on the concentrations of these drugs found in urine as this information is severely lacking in the scientific literature. In addition, this data will also be useful to toxicological investigations.

The results will have no impact on your legal proceedings.

Why have I been chosen?

You have been chosen, as you are required to provide a urine sample under your drug court order; this makes you a suitable candidate for this study.

Do I have to take part?

Taking part is up to you. If you do decide to take part you will be given this information sheet to keep, asked to sign a consent form and complete a questionnaire. You may withdraw from the study at any time, see the Contact for further information section overleaf. The results will have no impact on your legal proceedings.

What will happen to me if I take part?

To take part in this study, you are asked to consent freely to have your urine sample tested for drugs of abuse including NPS. The urine sample will be taken from the one you provided for your drug court order. You will not be asked to provide an additional sample. The urine sample will be anonymised and only your nurse will hold personal details. The results will have no impact on your legal proceedings.

Will my taking part in this study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. No individual results will be communicated to third parties. Your anonymised results along with other participants will be published in peer-reviewed papers and presented at international conferences.

What will happen to the results of the research study?

Any publications resulting from this study will not contain any indentifying information and will deal with the combined results from all participants.

Who is organising and funding the research?

This study is organised by the Forensic Medicine and Science unit of the University of Glasgow and is funded by the bench fees of Lauren O'Connor and NHS Greater Glasgow and Clyde Alcohol and Drug Health Improvement Team.

Who has reviewed the study?

This study has been reviewed by an independent group, the College of Medical, Veterinary and Life Sciences Research Ethics Committee, University of Glasgow.

11. Contact for Further Information

For further information concerning this study please contact Lauren O'Connor at Lauren.O'Connor@glasgow.ac.uk

To withdraw from the study please contact Lynn MacDonald at the Glasgow Drug Court on 0141 274 6000 or at lynn.macdonald2@ggc.scot.nhs.uk

Thank you for participating in this study.

If you have any concerns regarding the conduct of this research project, you can contact the College of MVLS Ethics team, email: mvls-ethics-admin@glasgow.ac.uk

11.5. Scottish Drug Court Participants Consent Form

University of Glasgow | Section of Forensic Medicine & Science

ID no.

CONSENT FORM

Detecting drugs of abuse including NPS in Urine Samples

Name of Researcher: Lauren O'Connor

Please initial box

I confirm that I have read and understand the information sheet dated _____ for the above study and have had the opportunity to ask questions.	☐
I understand that my participation is voluntary and that I am free to withdraw at anytime, without giving any reason, without my legal rights being affected.	☐
I agree to take part in the above study.	☐
I agree to my urine being tested for drugs of abuse including NPS.	☐

_____	_____	_____
Name of participant	Date	Signature
_____	_____	_____
Name of Person taking consent (Different from researcher)	Date	Signature

11.6. NHS GCC FD Participant Information Sheet

University of Glasgow
Forensic Medicine and Science

Participant Information Sheet

Prevalence of Novel Psychoactive Substances in Urine Samples Submitted for Toxicology Testing in Forensic Psychiatric Patients

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of the study?

The purpose of this study is to determine how effective the current NHS procedures are for testing urine samples for drugs of abuse, particularly novel psychoactive substances (NPS).

Why have I been chosen?

You have been chosen as you are currently a patient at an NHS Greater Glasgow and Clyde (GGC) Forensic Directorate facility.

Do I have to take part?

Taking part is completely up to you.

What will happen to me if I take part?

To take part in this study, you are asked to sign the consent form which will permit your urine sample to be tested for this research project. The urine sample will be anonymised and no individual involved in this study can or will identify you at any time.

University of Glasgow
Forensic Medicine and Science

Will my taking part in this study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. No individual results will be communicated to third parties. Your anonymised results along with other participants will be published in peer-reviewed journals and presented at international conferences.

What will happen to the results of the research study?

Any publications resulting from this study will not contain any identifying information and will deal with the combined results from all participants.

Who is organising and funding the research?

This study is organised and funded by Forensic Medicine and Science at the University of Glasgow in conjunction with the NHS GGC.

Who has reviewed the study?

This study has been reviewed by an independent group, the College of Medical, Veterinary and Life Sciences Research Ethics Committee, University of Glasgow, and by the Research Ethics Forum at the NHS.

11. Contact for Further Information

For further information concerning this study please contact Alice Turnbull at ForensicMedicine.Office@glasgow.ac.uk

Thank you very much for participating in this study.

If you have any concerns regarding the conduct of this research project, you can contact the College of MVLS Ethics team, email: mvls-ethics-admin@glasgow.ac.uk

11.7. NHS GCC FD Participants Consent Form

University of Glasgow
Forensic Medicine and Science

CONSENT FORM

Prevalence of Novel Psychoactive Substances in Urine Samples Submitted for Toxicology Testing in Forensic Psychiatric Patients

Name of Researcher: Alice Turnbull

Please initial box

I confirm that I have read and understand the information sheet dated_____ for the above study and have had the opportunity to ask questions.	☐
I understand that my participation is voluntary.	☐
I agree that my urine sample can be included in this study.	☐
I agree to my urine being tested for drugs of abuse including NPS.	☐

_____	_____	_____
Name of participant	Date	Signature
_____	_____	_____
Name of Person taking consent (Different from researcher)	Date	Signature

(Consent Form to be retained by NHS)

11.8. Scottish Drug Court Participant Questionnaire

University of Glasgow | Section of Forensic Medicine & Science

ID no.

Participant Questionnaire

Detecting drugs of abuse including NPS in Urine Samples

You have agreed to take part in the above research study. Please answer the following questions to the best of your ability. The more information you can give the better the results of the study will be.

All questionnaires are anonymised and will only be labelled with a number that will allow us to match them to the urine sample you have given permission for us to test. No identifiable information will be made available to any outside organisation.

Thank you for taking part.

Q1. What drug(s) (legal and illegal) have you taken in the last week?

1.

2.

3.

4.

5.

6.

7.

Others: _____

Q2. What did the drug(s) look like?

Tablet, powder, liquid, crystals, plant matter....

1.

2.

3.

4.

5.

6.

7.

Others: _____

ID no.

Q3. When was the last time you used the drug(s)?

1.
2.
3.
4.
5.
6.
7.

Others: _____

Q4. How much, of each, did you use at last use?

1.
2.
3.
4.
5.
6.
7.

Others: _____

Q5. How did you take each drug(s)?
Injected, snorted, smoked, swallowed.....

1.
2.
3.
4.
5.
6.
7.

Others: _____

Q6. Where did you purchase the drug(s)?
Internet, Dealer, Headshop, Friend, Market stall, corner shop
Other?
1.

2.

3.

4.

5.

6.

7.

Others: _____

Q7. Do you use the drug(s) regularly?
1.

2.

3.

4.

5.

6.

7.

Others: _____

11.9. Ethical approval to test the Scottish Prison Service urine samples.

WoSRES
West of Scotland Research Ethics Service

NHS
Greater Glasgow and Clyde

West of Scotland REC 3
Ground Floor – The Tennent Institute
Western Infirmary
38 Church Street
Glasgow G11 6NT
www.nhsggc.org.uk

Miss Alice Turnbull
Forensic Medicine and Science
Joseph Black Building
University of Glasgow
University Place
Glasgow
G12 8QQ

Date 9th October 2015
Your Ref
Our Ref
Direct line 0141 211 2123
Fax 0141 211 1847
E-mail WOSREC3@ggc.scot.nhs.uk

Dear Miss Turnbull

Study title:	**Prevalence of Drugs of Abuse Including Novel Psychoactive Substances in Prisoners at Scottish Prison Service Facilities**
REC reference:	**15/WS/0207**
IRAS project ID:	**190131**

Thank you for responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered in correspondence by a Sub-Committee of the REC. A list of the Sub-Committee members is attached.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Mrs Liz Jamieson, wosrec3@ggc.scot.nhs.uk.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Insurance or Indemnity Letter]		12 August 2015
IRAS Checklist XML [Checklist_08102015]		08 October 2015
Other [Confirmation To Seek Consent]		08 October 2015
Participant consent form [Participant Consent Form]	1	09 September 2015
Participant information sheet (PIS) [Participant Information Sheet]	2	05 October 2015
REC Application Form [REC_Form_11092015]		11 September 2015
Research protocol or project proposal [Research Proposal]	2	05 October 2015
Summary CV for Chief Investigator (CI) [CV AT]		
Summary CV for supervisor (student research) [CV HJT]		

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

15/WS/0207	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

Liz Jamieson

Liz Jamieson REC Manager
On behalf of Dr Adam Burnel, Chair

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*
"After ethical review – guidance for researchers"

Copy to: *Dr Debra Stuart, University of Glasgow*

11.10. Ethical approval for the data mining of post-mortem blood samples

University of Glasgow

29th July 2019

MVLS College Ethics Committee

Project Title: Investigation into Novel Psychoactive Substance (NPS) in post-mortem samples in Scotland

Project No: 200180192

Dear Dr Torrance,

The College Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study. It is happy therefore to approve the project.

- Project end date: End March 2020
- The data should be held securely for a period of ten years after the completion of the research project, or for longer if specified by the research funder or sponsor, in accordance with the University's Code of Good Practice in Research: (http://www.gla.ac.uk/media/media_227599_en.pdf)
- The research should be carried out only on the sites, and/or with the groups defined in the application.
- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.
- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely,

Jesse Dawson
MD, BSc (Hons), FRCP, FESO
Professor of Stroke Medicine
Consultant Physician
Clinical Lead Scottish Stroke Research Network / NRS Stroke Research Champion
Chair MVLS Research Ethics Committee

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